The immunological balance between host and parasite in malaria

Katrien Deroost[1,2], Thao-Thy Pham[1], Ghislain Opdenakker[1], Philippe E. Van den Steen[1]

[1] Laboratory of Immunobiology, Rega Institute for Medical Research, KU Leuven – University of Leuven, Belgium

[2] The Francis Crick Institute, Mill Hill Laboratory, Londen, UK

One-sentence summary:

The intricate balance between antimalarial immunity and parasite virulence factors including immune evasion mechanisms determine the outcome of malaria infections, as imbalances resulting in exaggerated parasite growth, excessive inflammation or the combination of both result in severe pathology.

Running title: The immunological balance of malaria

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Corresponding author:
Prof. Philippe E. Van den Steen
Laboratory of Immunobiology, Rega Institute for Medical Research, KU Leuven – University of Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium
Phone: +32-16-337363
Fax: +32-16-337340
E-mail: Philippe.vandensteen@rega.kuleuven.be

ABSTRACT

Coevolution of humans and malaria parasites has generated an intricate balance between the immune system of the host and virulence factors of the parasite, equilibrating maximal parasite transmission with limited host damage. Focusing on the blood-stage of the disease, we discuss how the balance between antiparasite immunity *versus* immunomodulatory and evasion mechanisms of the parasite may result in parasite clearance or chronic infection without major symptoms, whereas imbalances characterized by excessive parasite growth, exaggerated immune reactions or a combination of both cause severe pathology and death, which is detrimental for both parasite and host. A thorough understanding of the immunological balance of malaria and its relation to other physiological balances in the body is of crucial importance for developing effective interventions to reduce malaria-related morbidity and to diminish fatal outcomes due to severe complications. Therefore, we discuss in this review the detailed mechanisms of antimalarial immunity, parasite virulence factors including immune evasion mechanisms, and pathogenesis. Furthermore, we propose a comprehensive classification of malaria complications according to the different types of imbalances.

1. INTRODUCTION

Malaria remains a major health burden in tropical and subtropical countries. According to the annual World Malaria Report of the World Health Organization, an estimated 198 million people were clinically affected by malaria in 2013 and approximately 584 000 of these patients died due to severe complications. More than 90% of deaths occur on the African continent where mainly children and pregnant women are a risk of developing life-threatening complications. Adults in high-transmission areas have acquired semi-immunity against the parasite and are therefore protected against complications, but often harbor parasites as asymptomatic and transmitting carriers. In low-transmission areas, semi-immunity is less probable to develop and also adults are at risk of severe malaria [1]. Although five different *Plasmodium* species are known to infect humans, most of the infections are caused by *P. falciparum* and *P. vivax*. In the last 150 years, important progress was made in the understanding of malaria, and due to the availability of effective antimalarial drugs, the widespread use of vector control programs, increased funding and the improved functioning of local health systems, global malaria mortality rates have decreased by 47% since the year 2000. However, these progresses are challenged due to the development of resistance against the currently used antimalarial drugs and insecticides, the presence of asymptomatic but infective carriers, the decreased funding and the increased number of nonimmune travelers and refugees, and malaria-associated complications remain highly prevalent and lethal. Furthermore, if we want to further decrease morbidity and mortality or even go onto the path toward elimination, a better understanding of the mechanisms steering the immunological balance towards parasite clearance or severe complications is of paramount importance.

1.1. Multiple balances determine the outcome of malaria infections

Infection is initiated when sporozoites are injected together with anticoagulant saliva during a blood meal of an infected *Anopheles* mosquito. Parasites migrate to the liver in search of a favorable niche in the hepatocyte and successfully replicate in the liver, after which newly formed merozoites are released in the blood stream. During the blood-stage of the asexual developmental cycle, malaria parasites replicate inside red blood cells (RBCs). As RBCs are not targeted by cytotoxic cells, the parasite is temporarily protected from being eliminated. Nevertheless, the immune system is equipped to protect the host against an ongoing infection. While digesting host hemoglobin (Hb) and replicating inside the RBC, the parasite expands and changes RBC membrane deformability. *Plasmodium falciparum (P. falciparum)*-infected RBCs (iRBCs) become more rigid, whereas *P. vivax*-infected cells become more flexible [2]. The splenic macrophage phagocytotic system efficiently removes *P. falciparum*-iRBCs from the blood stream as they are not flexible enough to pass through the interendothelial slits of the sinuses [3, 4]. In addition,

individual merozoites, released into the circulation at schizont rupture, can be recognized by adaptive immune mechanisms and may be captured before invading new RBCs [5, 6].

The *Plasmodium* parasite has evolved survival strategies for immune evasion to sustain parasite replication until sexual gametocytes are picked up by a mosquito, thus supporting malaria transmission. While growing inside the iRBC, parasite antigens are inserted in the RBC membrane through a specialized transport system and interact with several adhesion molecules on the endothelial lining of the peripheral microvasculature [7, 8]. This phenomenon, which is called sequestration, retains the iRBCs containing the more mature forms (i.e. trophozoites and schizonts) in different organs such as lungs, brain, placenta, intestinal tract, spleen, bone marrow and adipose tissue, to evade the splenic sieving machinery. Furthermore, sequestration may contribute to create a metabolically favorable niche, or even to facilitate invasion as is suggested for reticulocyte-restricted parasites [9]. Because of these surface parasite adhesins, the iRBCs are no longer 'invisible' for the adaptive immune system and become the target of specific antibodies (Abs). To circumvent these adaptive immune mechanisms, the cell-surface proteins are subjected to antigenic variation, i.e. the parasite switches to other adhesin variants on the iRBC membrane and temporarily escapes the immune systems' vigilance. This is translated in wave-like parasitemia courses and recrudescences [10]. Furthermore, a high diversity of exposed proteins between isolates and the release of immunomodulatory parasite molecules in the circulation during schizont rupture further contribute to the inability of the host to mount sterile immunity.

The interplay between antiparasite immunity elicited by the host and virulence mechanisms of the parasite in some cases leads to severe pathology. A thorough understanding of the balance between the two systems is of crucial importance. Additional balances, i.e. metabolic, erythropoietic and vascular balances, may further complicate the outcome of infections (Figure 1). Therefore, we generated an extensive overview of the current knowledge about antiparasite immunity, parasite virulence factors resulting in immunomodulation or immune evasion, and pathological processes, after having considered some important aspects of parasite and host coevolution. Immune mechanisms are also activated during the liver stage of the disease, but since only asexual blood-stage parasites contribute to disease symptoms, we will only discuss the mechanisms related to the asexual erythroid development of the parasite. However, it should be kept in mind that the initial load of merozoites coming out of the liver might have an impact on the generation of immune responses and the subsequent (im)balances.

1.2. Evolution leads to a fine balance between parasite growth and immune control

Parasites take profit of their host by utilizing them as a food source, for protection against the environment and for transmission and spreading. In general, parasite virulence and high parasite densities

within a host are positively associated with increased transmission [11]. Excessive virulence rapidly kills the host and results in abrogation of parasite transmission. As a consequence, parasite virulence evolves according to this trade-off to an optimum with an 'intermediate' virulence. Since parasites evolve rapidly and adapt to changes in host or environment in only a few decades, partial immunity or the use of non-sterilizing vaccines may select for parasites with higher virulence in non-immune or non-vaccinated people [12, 13]. For malaria, this may also be applicable but it is further complicated by the fact that transmission is ensured by gametocytes, whereas virulence and pathology are more related to the asexual blood-stages. The asexual blood-stages are crucial to generate gametocytes, which do not multiply but may remain in the circulation for several weeks and may continue their life cycle when taken up by mosquitoes. The complex biology of gametocytes, the factors influencing the asexual stage-to-gametocyte conversion, the gametocyte prevalence and transmission to mosquitos are subject to intense research and have been reviewed [14].

The host is also evolving under selective pressure by parasites towards minimization of host damage, albeit a broader timescale is needed to induce these adaptive changes. Evidence that malaria is exerting substantial selective pressure on the human genome is provided by the multiple polymorphisms in genes encoding for RBC proteins [15, 16]. Some polymorphisms are harmful for the host but are maintained in the human population in malaria-endemic regions because of the partial resistance they provide against malaria. Some good examples of this are the sickle cell and thalassemia gene mutations in the genes encoding Hb. Furthermore, immune mechanisms to clear parasites are counterbalanced by immune evasion mechanisms of the parasite and, therefore, tolerance may appear, i.e. the ability of the host to minimize virulence without necessarily decreasing parasite density [17]. In turn, evolution to tolerance in the host results in a shift of the virulence trade-off optimum and may select for parasites with higher within-host densities and transmission. A certain level of tolerance to malaria parasites is evidenced by the higher pyrogenic threshold in adults [18]. Ultimately, coevolution may lead to harmless commensalism. This requires a delicate balance between a robust but tightly controlled immune response elicited by the host to eliminate the parasites without causing collateral damage and mechanisms used by the parasite to modulate or evade this immune response. Imbalances, i.e. insufficient immunity resulting in exaggerated parasite growth, exaggerated immune reactions and inflammation, or both, are harmful and in some patients may even lead to life-threatening complications. Throughout this review, this equilibrium will be referred to as the immunological balance (Figure 2A).

Malaria parasites presumably did not simply coevolve and differentiate together with their hosts, but rather appeared later in the evolution and spread to different hosts, including humans, by host switches [19]. This might in part explain the considerable disease burden by malaria in the human population. The

most plausible scenario for the origin of *P. falciparum*, which causes the greatest malaria burden in humans, is that it originates from gorillas [20], even if *P. falciparum* (-like) strains have also been detected in other primates [21-23]. Similarly, parasites closely related or identical to *P. vivax*, *P. ovale* and *P. malariae* have been identified in wild-living chimpanzees [20, 24]. The origin of *P. vivax* is most probably in African apes from where it spread to humans over the continents. Later, it disappeared from the human African population, most probably due to counterselection by the widespread Duffy-negative phenotype [25]. Analysis of the sequence diversity between different isolates suggests that *P. falciparum* appeared more recently in humans than *P. vivax*, a parasite which in general is less virulent than *P. falciparum* [26]. Furthermore, tolerance evolution in the main host, and a concomitant shift towards higher intrinsic virulence of a parasite, may explain the higher virulence of a parasite in non-adapted hosts, as is observed for severe zoonotic diseases [17]. This is illustrated by *P. knowlesi*-infections, which result from zoonotic transmission from macaques to humans in Southeast Asia and often cause severe disease [27].

The immunological balance is further complicated by genetic variations in both host and parasite, by the hosts' nutritional status, by additional infections with other microorganisms, and by the polyclonality of *Plasmodium* infections. When several parasite (sub)strains are present in a single host, competition for space and nutrients results in positive selection of more virulent strains, as has been shown experimentally in animal models [28]. In a population, high transmission is supposed to result in a high complexity of infection (polyclonality), leading to increased recombinations during the sexual phase in the mosquito [29]. Adaptive immune pressure by the host selects for low-frequency alleles in immune-exposed genes of the parasite and negatively selects against high frequency alleles, a process called balancing selection [30]. Such balancing selection prevents both fixation and loss of alleles and, therefore, leads to a high genetic diversity within the parasite population. Duplication of genes has generated multigene families displaying antigenic variation by differential expression and allelic exclusion, such as the *var* multigene family encoding *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1), the main mediator of cytoadherence and parasite sequestration [31]. The expression of these antigenic variant genes is also an important determinant for immune evasion and pathology and greatly influences the immunological balance.

A striking parallel example of this immunological balance is the disease course elicited by the three different genotypes of *Toxoplasma (T.) gondii* in mice, which match the three different outcomes of the immunological balance and of which the molecular basis has been elucidated in great detail [32]. *T. gondii* type 1 parasites induce an anti-inflammatory response and evade intracellular killing, resulting in death by hyperproliferation of the parasite. In contrast, *T. gondii* type 2 parasites rather induce an

exaggerated T-helper (Th)1 response resulting in immunopathology. Type 3 *T. gondii* activates anti-inflammatory signaling, does not evade intracellular killing, and is able to establish persistent and latent infections in mice.

As will be detailed below, in malaria, the immunological balance may also be tilted to one or the other side, depending on the host and parasite species and strain. In addition, the combination of high parasite burden and exaggerated immunopathology may also occur. Thus, it will be a challenge to eradicate a parasite with an evolutionary lifespan going back far beyond the origin of man and that has acquired several survival strategies and selected for protective genetic polymorphisms in the host in a process of coevolution.

1.3. Literature database and role of mouse models in malaria research

Defining the immunological balance in malaria patients is a major challenge. Because of concomitant inflammation and metabolic disturbances, it is difficult to discriminate between the different types of imbalances, i.e. insufficient immunity and/or excessive immune evasion promoting parasite replication, exaggerated inflammation resulting in immunopathology, or the combination of excessive immune evasion and exaggerated inflammation (Figure 2A). In patients, this is further complicated by genetic variation, environmental factors and therapeutic interventions. As such, mouse models of malaria have provided crucial insights in molecular events dictating these balances, because of inherent technical possibilities that are not available in clinical studies for ethical reasons. For example, with the use of inbred strains of mice it is possible to exclude genetic differences and, by microbiome monitoring, specific coinfections may be excluded. In human studies, this is not possible. Hitherto, commonly used methods to investigate immunological balances are mouse models with rodent malaria parasites (*P. berghei*, *P. chabaudi*, *P. yoelii* and, to a lesser extent, *P. vinckei*). The natural hosts of these parasites are African tree rats (*Grammomys surdaster* and *Thamnomys rutilans*) and these parasites are phylogenetically somewhat more related to *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* than to the Laverania, the *Plasmodium* subgenus that includes *P. falciparum* [33, 34]. Rodent and human malaria species are different and, therefore, experimental malaria infections in rodents are not perfect models of human malaria. For example, the acquisition of immunity in mouse models of malaria may already occur after a single infection, whereas this may not be the case in humans due to the high diversity of the different circulating (sub)strains. Nevertheless, mouse models are excellent tools to dissect the molecular details of the immunological balance and provide the possibility of testing interventions to restore imbalances. When extrapolating such data to possible treatments for patients, it is of crucial importance to

understand the immunological (im)balances in both animal models and in patients to avoid misinterpretation and adverse outcomes in clinical trials.

Extensive data are available in the literature on the molecular immunology of malaria, both from clinical and rodent studies. To generate a complete overview, we constructed a database containing over 2450 entries from more than 500 PubMed publications (www.malarimdb.org). The practical use of this database has been discussed in detail [35]. In this database, levels of diverse immunological factors are schematically compared in patients with different degrees and complications of malaria, and also expression data and experimental treatments or knockout studies in mouse models are included. The database is fully searchable and allows the comparison of patient and mouse data. The main insights from this database form the basis for the construction of this manuscript.

2. Antiparasite immunity

Upon a primary infection, an immune response is generated in which the spleen plays a central role [4, 36]. Innate immune mechanisms are first activated and limit the acute phase of parasitemia but are not able to clear the infection. Adaptive immunity is activated when dendritic cells (DCs) present processed antigens to naïve T cells. Activated T cells then provide help to Ab-producing cells. Eventually, the infection may be cleared from the circulation by Ab-mediated immunity directed against specific parasite antigens, unless immune evasion mechanisms by the *Plasmodium* parasite provide sufficient counterbalance against immune clearance. In this section, we discuss the mechanisms of antiparasite immunity. In section 3, the parasitic virulence factors and immune evasion mechanisms which interfere with the generation of efficient antiparasite immunity will be detailed, and section 4 is an extensive overview of how imbalances result in severe pathology. The influence of immunoregulatory mechanisms, elicited by the parasite or mounted by the host to counter inflammation, dampen inflammatory damage but also antiparasite immunity and these have therefore a crucial influence on the immunological balance between parasite and host (see section 5). Based on these insights, we attempt to classify the various outcomes and complications according to this balance in section 6 and summarized this in Figure 2 (panels B and C).

2.1. Direct killing mechanisms in malaria

In this section, the direct effector mechanisms which remove the pathogen from the circulation will be discussed, i.e. phagocytosis, complement, antibodies and cytotoxicity. Section 2.2 will cover indirect mechanisms that govern, improve and specify the effector functions. This includes cytokine production, antigen presentation to activate $CD4^+$ T cells and B cell activation.

2.1.1. Phagocytosis

Phagocytosis is a process by which foreign antigens or particles such as iRBCs, free merozoites, and hemozoin (Hz) are recognized, captured, internalized and neutralized by specialized cells of the immune system like monocytes, macrophages, polymorphonuclear granulocytes (PMNs) or DCs. It plays an essential role in parasite clearance [5, 37-40]. Phagocytic removal of malaria parasites predominantly occurs by macrophages in spleen and liver, as witnessed by the large amount of pigment found in these organs [41-43]. During an acute malaria infection, monocytes are massively recruited from the bone marrow, and contribute, in addition to local proliferation, to the hepatosplenomegaly so typically observed in malaria patients and mice [41, 44]. Once infiltrated in the tissues, these monocytes differentiate into macrophages or DCs, and augment the organs' phagocytic and antigen presentation capacity. In between bone marrow and the target organs, monocytes travel through the peripheral circulation. Depending on the maturation status, different blood monocyte subsets can be distinguished, which differ in their cytokine secretion and their capability to phagocytose merozoites, iRBCs and/or pigment. For example, the $CD14^{hi}$CD16/Fcγ receptor (FcγR) $IIIA^{+}$ 'inflammatory' or intermediate monocyte subset, followed by the more mature 'macrophage-like' $CD14^{lo}$CD16/FcγR$IIIA^{+}$ subset, seem to be the monocyte subtypes primarily equipped for phagocytosis of *P. vivax*-infected cells *in vitro* [45]. In *P. falciparum*-infected children, the expression level of CD16/FcγRIIIA on these two subsets correlates with tumor necrosis factor (TNF) production and in case of the more mature $CD14^{lo}$CD16/FcγR$IIIA^{+}$ subset, a negative correlation was found with Hb levels, thereby suggesting a role in erythrophagocytosis [46].

Phagocytosis is initiated when pathogen-associated molecular patterns (PAMPs) of parasitic origin interact with pattern-recognition receptors (PRRs) on the host cell surface. Parasite antigens interact with a wide range of different receptors, and the relative importance of particular receptors changes during the course of infection. In the early phase of antimalarial immunity, the scavenger receptor CD36 seems to be the main receptor involved in parasite removal, both in humans and in mouse models [47-50] (Figure 3). Interaction between parasite-derived products and Toll-like receptors (TLRs) or mannose receptors have also been described [51-53], but might be more important for the subsequent signaling they induce than for parasite removal per se [47, 53-57]. Furthermore, the complement system is activated and produces soluble complement factors that can act as opsonins. When complement-bound parasite antigens interact with complement receptors (CRs) on phagocytes, they are subsequently phagocytosed [40, 58]. As the adaptive immune system is not yet fully active, parasite replication exceeds parasite clearance, and thus the initial phase of a blood-stage malaria infection is one of increasing asexual parasitemia. Later during

the infection, specific Abs are secreted that also act as opsonins and mediate phagocytosis via binding to FcγRs [58-60]. Opsonophagocytosis mediated by Abs is antigen-specific and accelerates the interaction between free merozoites or iRBCs and phagocytes and reduces peripheral parasitemia.

Once the parasite is bound to the phagocyte, the receptor-parasite complex is internalized by an actin-dependent process [39]. The phagosome fuses with lysosomes to form a phagolysosome with an acidic pH that is ideally suited for protein complex dissociation and degradation by acidic proteases [61]. Except for Hz, the crystalline waste product of Hb consumption which remains intact inside the phagocyte, all parasite constituents are efficiently degraded and the used receptors are recycled. Depending on the type of receptor used for binding and internalization, phagocyte activation is triggered and translated in an increased expression of FcγRs and CRs, generation of a respiratory burst and secretion of proteolytic enzymes and cytokines [40, 53, 62, 63]. Cytokines orchestrate the antiparasite immune response but are also involved in severe pathology (see further). Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) produced during the respiratory burst are cytotoxic for the internalized parasites and can cross the cell membrane to cause damage to merozoites and iRBCs that are in close proximity to the monocyte/macrophage [64]. Free radicals generated by the xanthine oxidase/hydrogen peroxide system and NADPH oxidase in combination with inducible nitric oxide synthase (iNOS), seem to be primarily involved in inducing the degeneration of intraerythrocytic parasites into crisis forms [45, 64-66]. PMN activity against adherent parasites has also been demonstrated, but seems to be ROS-independent [67, 68].

2.1.2. Activation of the complement system

The complement system is activated early during infection. Mannan-binding lectin (MBL), an acute-phase protein produced by the liver, can activate the complement system through the lectin pathway and has been shown to bind carbohydrate structures on iRBCs [69, 70]. Ab-parasite-complexes can activate the classical pathway. In particular, Ab-mediated fixation of complement on merozoites results in the formation of the membrane attack complex and this plays a major role in the Ab-mediated inhibition of invasion [71]. Free digestive vacuoles containing Hz and band 3 receptor clustering can trigger alternative complement pathway activation [72-74]. Both senescent and malaria-infected erythrocytes suffer from increased oxidative insults. This results in increased hemichrome deposition and promotes band 3 receptor clustering on the RBC membrane. In this way, new epitopes are exposed, recognized by naturally-occurring anti-band 3 auto-Abs with subsequent C3b deposition, and these C3b-coated immune complexes (ICs) are recognized by CR1 (CD35) resulting in erythrophagocytosis. Increased levels of anti-band 3 class G Immunoglobulins (IgGs) are detected in serum of *P. falciparum*-infected children [75, 76]. This type of phagocytosis is accelerated in the presence of underlying erythrocyte disorders such as sickle cell trait, beta-thalassemia, glucose-6-phosphate dehydrogenase (G6PDH) deficiency, and pyruvate kinase

deficiency [77]. Furthermore, it is influenced by the ABO phenotype and might in part explain the predominance of blood group O in malaria-endemic regions. Hemichrome formation and band 3 aggregation is increased in ring and mature-stage infected O-type RBCs compared to A or B type RBCs, and O type iRBCs are more avidly phagocytosed [78].

Besides phagocytosis, complement-mediated lysis may also occur. iRBCs are relatively resistant towards complement-dependent lysis by the presence of complement regulatory proteins on the plasma membrane of human iRBCs [e.g. CR1, decay accelerating factor (DAF or CD55) and protectin (or membrane inhibitor of reactive lysis (MIRL) or CD59)] [79, 80]. This can be circumvented by binding of C-reactive protein (CRP), an acute-phase protein that is increased in plasma of malaria-infected patients, to the red cell membrane [80-83]. CRP binds to the surface of apoptotic cells, and phosphatidylserine (PS) exposure, a characteristic feature of apoptotic cells, has been observed on the outer surface of iRBCs [84-86]. The interaction between CRP and RBCs decreases the expression and the affinity of complement-regulatory proteins and favors complement-deposition [80].

The precise strategy how the complement system acts during antimalarial immunity i.e. by opsonophagocytosis or complement-dependent lysis, has not yet been fully clarified. In general, however, the complement system seems to play only a minor role in the resolution of a primary infection because even in the absence of complement, parasites are cleared from the circulation [87, 88]. In contrast, exaggerated complement activation has been observed in severe malaria patients and contributes significantly to pathology, including the promotion of inflammation and anemia [75, 89, 90]. Furthermore, the terminal part of the complement pathway is activated in mice with cerebral malaria (CM) as illustrated by increased C5 levels and C9 deposition in mouse CM brains [91, 92], and deposition of C3d and the assembled C5b-9 complex on sequestering RBCs in brain sections of patients who died from CM [90].

CR1 has multiple roles in malaria, both beneficial and pathogenic. Besides mediating the removal of complement-decorated senescent and iRBCs, it is the main receptor involved in clearance of circulating ICs. If not removed, ICs accumulate in the circulation and in the kidney with glomerulonephritis as a result. Circulating ICs can interact with CD16/FcγRIII resulting in phagocyte activation and augmentation of the inflammatory response. Both in patients and in mice with malaria, CR1 surface expression is reduced on monocytes, macrophages, and B cells (but not on PMNs) resulting in increased levels of circulating ICs [93]. Furthermore, by the presence of CR1 on the surface of human RBCs, RBCs participate in IC clearance by transferring CR1-bound ICs to phagocytic cells [93]. In this process, CR1 is lost from the red cell membrane. Since CR1 protects the red cell against complement deposition, this

mechanism can promote complement-mediated lysis of both infected and uninfected RBCs, and contribute to anemia (see section 4.2.5). CR1 and DAF/CD55 are also used by *P. falciparum*-merozoites to invade erythrocytes [94-96], and CR1 is also involved in immune evasion by mediating rosette formation of *P. falciparum*-iRBCs, i.e. clustering of iRBCs with uninfected RBCs [97] (see also section 3.3.1.2). Murine erythrocytes do not have CR1 but instead express a CR1-like molecule, Crry, which does not participate in clearance of circulating ICs [98].

2.1.3. Antibodies

During a primary infection, Ab-independent immune mechanisms are often able to limit the severity of infection. However, for complete parasite clearance, Ab-dependent immunity is indispensable as illustrated by the following observations. Malaria-infected B cell-deficient mice develop a low chronic parasitemia with higher recrudescences [99-103]. This B cell-deficiency may be compensated by an expansion of γδT cells, but, if the latter are also depleted, a high parasitemia persists [104, 105]. Antigen variation may occur with parasite antigens under high Ab pressure (see section 3.2.2). Therefore, the repertoire of specific Abs is broadened with subsequent *Plasmodium* infections [106]. The initial Ab repertoire may presumably protect against severe disease and broadened Ab repertoires are necessary against symptomatic infection and are a prerequisite for developing semi-immunity or premunition in malaria-endemic regions [[107, 108] and reviewed in [1, 109, 110]]. Passive transfer of purified IgGs from semi-immune people or from semi-immune mice (partially) suppresses peripheral blood parasitemia and symptoms as rapidly as antimalarial drugs [59, 111-113].

The isotype of antibodies produced does not only depend on the mode of B cell activation, e.g. extrafollicular or in germinal centers (GCs) (see section 2.2.3), but also on the inflammatory environment surrounding the B cell subsets. The proinflammatory Th1-type environment during malaria infection will predominantly stimulate isotype switching to cytophilic or opsonizing Abs such as IgG1 and IgG3 in humans or IgG2a and IgG2b in mice, which will be of main importance for controlling the infection through several mechanisms [114, 115], some of which are outlined here. Abs against merozoite proteins involved in RBC invasion, including merozoite surface protein-1 (MSP-1), apical membrane antigen-1 (AMA-1), and erythrocyte-binding antigen-175 (EBA-175), have been documented in sera from *P. falciparum*-infected individuals [116-119]. Invasion-inhibitory Abs recognizing *P. falciparum* reticulocyte-binding-homologue-4 (*Pf*Rh4) or *P. vivax* Duffy-binding protein (*Pv*DBP) correlated with protection against *P. falciparum* and *P. vivax* infections, respectively, in individuals from Papua New Guinea [120, 121]. Ab-mediated opsonic phagocytosis of merozoites correlates with protection from clinical episodes and high-density parasitemia in *P. falciparum*-infected children from Papua New Guinea

and Africa [122, 123]. Furthermore, Abs on merozoites activate the classical complement pathway, which triggers merozoite lysis, and this appears to be the most efficient mechanism for blocking reinvasion [71]. Once RBC invasion is successful, the parasite resides inside a protective parasitophorous vacuole shielded from the host cell cytoplasm. During maturation, the parasite exports parasite-encoded proteins to the iRBC membrane that enables the parasite to evade specific immune mechanisms (see section 3.3). Abs against these parasite proteins are involved in opsonic phagocytosis of iRBCs and interfere with endothelial cytoadherence, and/or mediate rosetting [5, 124-127]. Furthermore, inhibition of parasite growth by entering of Abs inside the iRBC has been described and may be enhanced by monocytes in a process of Ab-dependent cellular inhibition mediated by FcγRII and TNF [128, 129].

The malaria parasite has developed multiple strategies to interfere with and evade Ab-mediated immunity. These strategies include antigen variation and antigen diversity (section 3.3.2), and modulation of B cell maturation and the memory compartment (section 5.5). Consequently, with *P. falciparum* infections this type of immunity wanes rapidly and the acquisition of parasite-specific memory B cells (MBCs) and long-lived Ab-secreting cells is inefficient and slow [106, 130].

2.1.4. Cell-dependent cytotoxicity

Direct cell cytotoxicity can contribute to remove pathogens from the host in various infectious diseases. However, whether and to what extent this mechanism is also involved in the antimalarial response is not yet fully clear. Mature RBCs have lost the ability to express major histocompatibility complex (MHC) class I molecules and as a consequence are not subjected to $CD8^+$ T cell-mediated cytotoxicity. Several *Plasmodium* species, including *P. vivax* and particular strains of *P. yoelii* and *P. berghei*, preferentially infect reticulocytes above normocytes (see section 3.1). Reticulocytes express MHC class I molecules on their surface [131], but even in this case, MHC class I-dependent cytotoxicity by $CD8^+$ T cells does not seem to contribute to clearance of infected cells [132, 133]. Nevertheless, parasite-specific cytotoxic T lymphocytes (CTLs) are activated during malaria infection and contribute to the overall antiparasite response, both in the acute and in the chronic phase [134-138]. Their importance could be masked by the influence of the inhibitory programmed death-1 (PD-1) receptor, which seems to reduce considerably $CD8^+$ T cell numbers and functionality during malaria [136]. How CTLs contribute to parasite clearance remains elusive in the absence of MHC class I. Infection of erythroblasts, erythroid precursor cells, has been shown *in vitro* with *P. falciparum* and *in vivo* in the bone marrow of two *P. vivax*-infected but aparasitemic patients and also in *P. yoelii*-infected mouse spleens [138-141]. Erythroblasts express high levels of MHC class I and Fas, a receptor involved in apoptosis. Indirect killing of infected erythroblasts is suggested to occur through Fas-Fas ligand (FasL) interaction [138]. This results in PS disposure on the outer membrane of the cell, and accelerates engulfment of the apoptotic cell by phagocytes, possibly

mediated through T cell immunoglobulin-domain and mucin-domain-containing molecule 4 (Tim-4). The importance of this mechanism for parasite clearance seems limited, since even if erythroblasts are infected, their percentage is very small compared to infected erythrocytes and reticulocytes, which generally do not express Fas. This process can, however, contribute to ineffective erythropoiesis and may predispose to anemia.

Platelets most probably contribute to parasite clearance. Human and murine platelets secrete platelet factor 4 (PF4/CXCL4) from intracellular α-granules upon platelet activation. After binding to and internalization by the Duffy-Ag receptor (DARC/Fy/CD234) on iRBCs, the digestive vacuole of the parasite is lysed. This results in intraerythrocytic parasite killing and limits parasite replication before adaptive immunity is activated [142, 143]. These data implicate that drugs that inhibit platelet activation such as aspirin, which is often used before admission to the hospital, have potentially harmful effects during malarial infections [144]. Thrombocytopenia, which is reported in a large percentage of malaria patients, may also negatively influence this type of parasite clearance [145]. Furthermore, platelet-mediated killing is abrogated in DARC-deficient RBCs, indicating that this mode of protection against malaria may be absent in the vast majority of African inhabitants who are Duffy-negative and who suffer from the highest mortality rates due to *P. falciparum* malaria [142].

Natural killer (NK) cells are also activated during malaria. However, in mice, NK cells mainly contribute to the early resistance against a blood-stage infection by their cytokine secretion rather than their cytotoxicity (see section 2.2) [146, 147]. In humans, both mechanisms may contribute, since human NK cells are cytotoxic for *P. falciparum*-iRBCs *in vitro* (although contradictory data exist) [148-153], an effect that was dependent on granzyme B and Fas [151]. Recent data from immune cell-optimized humanized (RICH) mice now confirm the involvement of NK cell cytotoxicity for the control of *P. falciparum* parasitemia, an effect that was dependent on lymphocyte function-associated antigen-1 (LFA-1) and partly also on DNAX Accessory Molecule-1 (DNAM-1/CD226) [154].

The γδT cell subset may also inhibit *P. falciparum*-iRBC growth *in vitro* by killing free merozoites, an effect that seems to depend on granulysin [132, 155].

2.2. Immunological support mechanisms

Several mechanisms indirectly contribute to the removal of parasites from the blood. Cytokines are secreted by many different cells of the immune system and regulate the type and intensity of the host immune response (Figure 3). Early in the course of infection, when adaptive immunity is not yet activated, mainly phagocytes, NK cells, endothelial cells (ECs), γδ T and γδ NKT cells produce cytokines

to augment the required inflammatory response. Later, when specific αβ CD4 T helper (Th) cells and αβ NKT cells are present, this response is down- or up-regulated, depending on the polarization of the Th cell subsets present, and highly specific Ab-producing B cells are boosted. For the successful generation of the latter processes, optimal function of the splenic white pulp is of major importance, since the white pulp is the site where cells of the innate and adaptive immune system interact during malaria, and thus where the generated immune response is improved and specialized [36]. Disorganisation of the splenic architecture may therefore disturb antimalarial immunity and is discussed in more detail in section 5.5.

2.2.1. Cytokines orchestrate immune cell homeostasis and effector functions

Chemokines, cytokines and their respective receptors contribute significantly to the recruitment and activation of different cell types of the immune system and augment the phagocytic response. For optimal clearance of merozoites and freely circulating and sequestering iRBCs, sufficient phagocytes must be available in the circulation, spleen, liver, bone marrow and placenta, i.e. all organs where active phagocytosis occurs. Furthermore, adequate numbers of viable and activated DCs must be present in the spleen and other secondary lymphoid organs to stimulate adaptive cellular and humoral immune responses. Granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage (GM)-CSF in combination with interleukin-3 (IL-3) stimulate the proliferation and differentiation of myeloid precursors in the bone marrow [156-158]. Fms-related tyrosine kinase 3 ligand (Flt3L) produced by mast cells in response to uric acid release by iRBCs further augments bone marrow myelopoiesis [159]. The newly produced bone marrow-derived mononuclear cells are recruited to the circulation in a CC chemokine receptor 2 (CCR2)-dependent or independent way and fuel the phagocytic and antigen-presenting capacity of the spleen by increasing the number of inflammatory monocytes and $CD8\alpha^+$ myeloid DCs (mDCs). This in turn can augment interferon-γ (IFN-γ) production by NK cells and $CD8^+$ T cell activation [159-161]. Also in malaria-infected patients, highest circulating levels of $BDCA3^+$ DCs, the equivalent of $CD8^+$ mDCs in mice, and activated $CD8^+$ T cells, are found in patients with highest Flt3L levels [159].

Apart from a role in cell recruitment, cytokines also enhance the immune response. For instance, GM-CSF and TNF synergistically increase FcγR and CR expression on human neutrophils and, thereby, stimulate opsonin-dependent phagocytosis [162]. A central role is played by IFN-γ. Among others, IFN-γ induces myelopoiesis in the bone marrow, enhances the respiratory burst and cytokine secretion during the phagocytic process and, later during infection, IFN-γ augments the antigen-presenting activity of DCs, monocyte/macrophages and B cells resulting in activation and intensification of acquired T and B cell immunity [40, 163-166]. Therefore, Th1-type reactions are crucial to mount efficient antimalarial immunity. However, IFN-γ is also involved in severe pathological reactions (section 4.2.3) and

insufficiently balanced levels of this cytokine may also contribute to inadequate regulation of the B cell response (section 5.5).

Presence or absence of IFN-γ secretion by NK cells and γδ T cells 24 hours after infection seems to discriminate between self-resolving and lethal *Plasmodium* infections in mice [146, 167, 168]. These cell types may also be involved in early IFN-γ responses in human infections, since *in vitro* incubation experiments with *P. falciparum*-iRBCs and human peripheral blood mononuclear cells (PBMCs) have indicated that IFN-γ is rapidly produced by NK cells, γδ T cells (including γδ NKT cells) and αβ T cells, although the relative contribution of the different cell types varies between individuals [152, 169, 170]. *Pf*EMP-1 decreases early IFN-γ responses (but not cytotoxicity) in NK cells, γδ T cells and αβ T cells, indicating that the parasite can modulate early host inflammatory responses [171]. In mice, activated DCs (and other myeloid-derived cells like monocytes/macrophages) can stimulate IFN-γ production by NK cells through direct cell-cell contact and secretion of soluble mediators including IL-12 [161, 172]. Concomitantly, NK cell-derived IFN-γ favors IL-12 production by DCs instead of IL-10. This NK cell-mediated induction of IL-12 seems also necessary in $CD8\alpha^+$ mDCs for the generation of parasite-specific $CD8^+$ T cells that are pathogenic in the brain of mice with CM (see section 4.2.3) [173]. IFN-γ production by human NK cells depends on IL-2, IL-18 and intercellular adhesion molecule-1 (ICAM-1)/LFA-1 ligation with monocytes/macrophages [153, 174].

Later during peak parasitemia, IFN-γ-producing $CD4^+$ T cells, together with activated NKT cells, will take over IFN-γ production from NK and γδ T cells and will become the major source of IFN-γ production in mice. A similar shift is noted for IFN-γ production in response to *P. falciparum*-iRBCs, i.e. the very early response is dominated by NK cells, whereas T cells become increasingly important at later time points [170]. This mechanism may be seen as a central theme during malaria infections, although differences may exist for the different parasite strains. For example, in rodent malaria infections with the nonlethal strain of *P. yoelii* (17XNL), an innate macrophage-mediated immune response that is independent of IFN-γ, NK cells or T cells and dependent on monocytes or macrophages seems to control the acute infection stage [175].

2.2.2. Antigen-presenting cells activate T cells and determine their polarisation

Antigen-presenting cells (APCs) provide an important link between innate and adaptive immunity. Different cell types can present exogenous protein antigens to conventional T cells in a MHC class II

restricted way (or glycolipid antigens to NKT cells in the context of CD1d). However, mainly DCs stimulate the differentiation and polarization of naïve T cells into the different Th cell subsets [161]. Stromal cell-derived factor-1 (SDF-1 or CXCL12) production early during infection supports DC homing to the splenic compartment and stimulates optimal positioning of DCs for antigen presentation to naïve T cells [176, 177].

In murine malaria models, classical or mDCs, but not plasmacytoid DCs (pDCs), seem to determine the type, magnitude and kinetics of the T cell response [172, 178]. Furthermore, pDCs seem to support the infection, survival and replication of murine *Plasmodium* parasites, and these 'infected' pDCs are infectious when transferred to naïve mice [179]. Maturation of immature DCs is shown to occur after direct cell-cell contact and phagocytosis of schizont-stage iRBCs and results in upregulation of MHC class II and costimulatory molecules (CD40, CD80 and CD86) and in IL-12 and TNF production [53, 180]. This maturation needs to be perfectly balanced, since a "hypermaturation" phenotype will block DC function as is shown for lethal malaria infections in mice characterized by high TNF levels [181]. In the spleen, activated DCs (but not macrophages or B cells) migrate from the marginal zone into the T cell rich areas and present their processed antigens in a MHC class II context to naïve T cells (Th0) [182]. In the presence of costimulatory molecules and IL-12, and in the absence of IL-10, a strong proinflammatory response (Th1) is induced with mainly IFN-γ-producing $CD4^+$ T cells.

The type of DC involved in activating adaptive immune responses in human infections is less clear, since only circulating DCs can be studied. Patients with uncomplicated *P. falciparum* or *P. vivax* infections have reduced numbers of both mDCs and pDCs and a decreased mDC/pDC ratio in peripheral blood, whereas immature DCs seem to accumulate. Furthermore, DC viability, maturation and functionality are impaired [183-186]. The impaired DC response could result from the increased Treg numbers and high IL-10 levels also observed in these patients. Tregs can alter the balance between circulating mDCs and pDCs, whereas IL-10 seems to be responsible for DC apoptosis. Nevertheless, *in vitro* experiments with *P. falciparum* indicate that partially activated mDCs are able to induce IFN-γ production by $CD4^+$ T cells [186] and, despite inadequate numbers of fully-activated circulating DCs, IFN-γ-producing $CD4^+$ T cells are observed in *P. vivax* and *P. falciparum* infection [187-189]. The regulation and suppression of the immune response is further discussed in section 5.

Interestingly, also platelets can process and present antigens in the context of MHC class I together with the necessary costimulatory molecules to promote T cell responses [190]. Vaccination of malaria-infected mice with *in vitro* stimulated platelets decreased parasitemia and subsequently protected against CM [190].

2.2.3. Generation of antibody-producing cells

In contrast to monocytes/macrophages that recognize specific motifs, i.e. PAMPS, B cells recognize specific conformational epitopes. It is a major function of $CD4^+$ T cells to stimulate B cells to produce Abs which eliminate the malaria parasites through the mechanisms outlined in section 2.1.3. T cell-dependent B cell activation occurs through both direct cell-cell contact and secretion of soluble mediators. Furthermore, the cytokines produced during infection also direct the magnitude and type of the acute humoral response.

Upon an encounter between a naïve B cell and its specific antigen, and in the context of extrafollicular T cell help, the B cell proliferates and differentiates into a short-lived Ab-producing plasma cell (PC) that secrete the Abs necessary to eliminate the pathogen during the acute infection. These short-lived PCs undergo apoptosis after a few days or weeks. Upon sustained antigen-specific stimulation with T cell help in germinal centers (GC), long-lived PCs and MBCs with a high affinity are produced. MBCs can circulate throughout the body and, when activated in the marginal zone or in GCs during a new attack with the same pathogen, they rapidly differentiate into Ab-producing PCs. Long-lived PCs travel to the bone marrow from where they constantly secrete low levels of Abs to form a first line defense during a second infection [191, 192].

During a GC reaction, interaction with follicular DCs (fDCs) and follicular $CD4^+$ T helper (Tfh) cells assists in selection of the clones with the highest affinity for the antigen, and only these high affinity B cell clones undergo class switching and differentiate into Ab-secreting PCs and MBCs. The process of affinity maturation improves the affinity by which the Ab binds to its antigen and also depends on the local presence of specific cytokines. In C57BL/6 mice infected with *P. chabaudi* AS or with *P. yoelii* 17XNL, IL-21 production by Tfh cells and interaction of IL-21 with its receptor on activated B cells appear to be crucial for the GC B cell reaction, for the generation of parasite-specific IgGs and to control parasitemia in the chronic phase of the infection or upon reinfection with the same parasite strain [193]. Interestingly, excessive IFN-γ levels may limit survival or differentiation of Tfh cells and thereby impede GC reactions. Therefore regulation of IFN-γ by inhibitory coreceptors plays an important role for optimal GC reactions [194] (see also section 5).

In order for the different B cell subsets to proliferate, differentiate and survive, they require B cell activating factor (BAFF) (reviewed in [191]). BAFF production is stimulated in activated myeloid cells such as monocytes and DCs, e.g. by IFN-γ and IL-10, after which it is exposed on the surface and then enzymatically cleaved and released as a soluble form. The type of BAFF (membrane-bound or soluble)

and the receptor used for this BAFF-signalling differs between naïve B cells, PCs and MBCs allowing them to be differentially stimulated. Both membrane-bound and soluble BAFF are produced early during human experimental infection and BAFF and BAFF receptor (BAFF-R) levels correlate with B cell activation [195]. Also in naturally infected children from malaria-endemic regions increased BAFF levels are observed [196]. However, most children had low expression levels of BAFF-R on peripheral B cells and only children with high B cell BAFF-R expression maintained parasite-specific IgGs over a few months time period, indicating the dysregulated BAFF signaling contributes to short-lived Ab responses. Loss of membrane-bound BAFF by mDCs has also been observed in *P. yoelii*-infected mice in which it negatively influenced the survival of differentiating MBCs. The parasite can also modulate this B cell response, since soluble *P. falciparum*-Ags and purified Hz can induce BAFF production by monocytes *in vitro* and BAFF levels correlate with peripheral parasitemia [195, 197]. Other influences of malaria on B cell-mediated immunity, resulting in atypical B cells and possibly B cell exhaustion are discussed in section 5.5.

3. Parasite virulence factors disturb the immunological balance by modulating or evading the host immune response

As discussed in the previous sections, the immune system of the host is well equipped to clear malaria parasites from the circulation. However, parasite replication results in the production and release of various parasite and RBC molecules that are immunologically active or even toxic and that modulate the induced immune response (Figure 4). Furthermore, the parasite has developed specific mechanisms, such as cytoadherence and antigenic variation and diversity, to evade antimalarial immunity. The interplay between the immunomodulating factors produced, the extent of immune evasion, and the host immune system determine the virulence of the infection and whether the immunological balance is in equilibrium or imbalanced. This section gives an overview of the parasite virulence factors that may interfere with the antimalarial immune response.

3.1. Intrinsic growth rate and host cell preference

A primary determinant of microbial virulence is the intrinsic growth rate. For blood-stage malaria parasites, this is determined by the number of merozoites produced per schizont and by the time required between RBC invasion and schizont rupture. The latter varies considerably between parasite species, i.e. 24h for *P. knowlesi* and the murine parasite species *P. berghei and P. chabaudi;* 36-48h for *P. falciparum*; 48h for *P. vivax and P. ovale* and 72h for *P. malariae*. In patients, this also corresponds to the cyclic febrile episodes. Parasites often mature and rupture synchronously thereby releasing high amounts of pyrogenic molecules during a narrow time-window, which triggers the production of endogenous

pyrogens such as TNF and IL-1β (reviewed in [198]). Cyclic temperature peaks promote intraerythrocytic growth, but high temperatures may kill the parasite [198]. Therefore, to protect itself from excessive heat, the parasite transports ring-infected erythrocyte surface antigen (RESA) proteins in the RBC cytoplasm to stabilize the spectrin tetramers of the RBC [199]. Also the number of merozoites per schizont is species-dependent. *P. falciparum*-schizonts produce the highest merozoite numbers (n = 16-36) compared to other species: *P. vivax* (n = 14-20), *P. ovale* (n = 6-18) and *P. malariae* (n = 8-12) [200]. Furthermore, with *P. falciparum* and *P. vivax*, a single erythrocyte can be infected by more than one parasite, although full parasite development may be hampered by the limited availability of nutrients and space. Maximally three merozoites may be able to mature asynchronously into schizonts inside a single erythrocyte [201].

Another major determinant of virulence is erythrocytic host cell preference. Invasion is reticulocyte-restricted for *P. vivax*, *P. ovale* and some rodent strains like *P. yoelii* 17XNL. This severely reduces the number of host cells available for invasion (typically < 1-2% of all RBCs) and limits parasitemia unless anemia drives reticulocytosis to higher levels. Moreover, *P. vivax* and *P. yoelii* 17XNL preferentially invade immature $CD71^{hi}$ reticulocytes, whose presence is largely restricted to erythropoietic tissues [202, 203]. *P. falciparum*, *P. knowlesi*, particular rodent parasites including *P. chabaudi* and some strains of *P. yoelii* and *P. berghei* also invade normocytes and, therefore, give rise to higher parasite loads.

The exact mechanism for reticulocyte-restricted tropism is not known. Binding of *P. vivax* and *P. knowlesi* to DARC is mediated by members of the Duffy binding-like family (DBLs), *Pv*DBP and *Pk*DBPα, respectively, and *P. vivax* reticulocyte binding proteins (*Pv*RBPs) [204-206]. However, DARC is also expressed on normocytes and this may therefore not fully explain the reticulocyte-restriction. For a long time, *P. vivax* was considered to be restricted to $DARC^+$ individuals, since the near absence of *P. vivax* malaria on the African continent coincides with the widespread presence of the Duffy-negative phenotype. Some *P. vivax* African hotspots exist, but this is due to the presence of Duffy-positive individuals. Lately, the incidence of *P. vivax*-infections is increasing in $DARC^-$ individuals, indicating that parasite virulence mechanisms are evolving slowly toward a DARC-independent reticulocyte invasion pathway [207, 208].

Interestingly, variations in parasite invasion molecules also influence host cell preference. For example, in the rodent malaria strain *P. yoelii,* each merozoite originating from a single schizont expresses a distinct variant of the *P. yoelii* 235-kDa rhoptry protein (PY235) multigene family, one of the reticulocyte binding protein homologues multigene families, to enable invasion of different erythrocyte subsets [209]. Notwithstanding, it seems that it is the amount and type of a specific PY235 variant expressed, but not the total repertoire of PY235 variants present, which determines host cell selection. However, when the most

abundantly expressed variant is disrupted in a lethal *P. yoelii* strain, the capacity to bind to mature erythrocytes is greatly diminished, and as such, also the number of host cells available for infection [210, 211].

3.2. Parasitic immunomodulatory factors

Several factors produced by the parasite or originating from the (rupturing) infected host cell, including GPI-anchors, Hz, heme, nucleic acids, microparticles (MPs) and exosomes have prominent immunomodulatory effects and substantially codetermine the type of immunological balance in the host and thus the outcome of the malaria infection. In this paragraph, the effects of these factors on the immunological balance are discussed.

3.2.1. Heme

Upon schizont rupture, residual Hb that was not digested by the parasite is released into the circulation. When insufficient plasma proteins are present to scavenge cell-free Hb, like hemopexin and albumin, Hb is oxidized into MetHb, which is highly unstable and eventually leads to the release of free heme (reviewed in [212]). Free heme is toxic and is suggested to play a role in the pathogenesis of murine CM. Heme in the presence of ROS might mediate blood-brain barrier (BBB) disruption and may cause neuroinflammation [213]. Furthermore, by activating the inflammasome heme triggers IL-1 production in macrophages [214]. Heme also downregulates anti-inflammatory mechanisms, by inducing the expression of Cu/Zn superoxide dismutase (SOD-1) in PBMCs, which inhibits prostaglandin E2 (PGE_2) and transforming growth factor beta (TGF-β) secretion [215]. This effect is partially mediated by CD14, since incubation of heme in the presence of anti-CD14 Abs reduces SOD-1 production and increases PGE_2, but not TGF-β secretion. Also Hz can inhibit PGE_2 secretion in *P. falciparum* patients [216]. PGE_2 is important to suppress TNF secretion by inhibiting cyclooxygenase-2 (COX-2), and, as such, protects against exaggerated inflammation. Induction of heme oxygenase-1 (HO-1) results in degradation of heme and the production of carbon monoxide (CO), which protects the host against cerebral complications by blocking $CD8^+$ T cell-mediated neuroinflammation [213]. In lethal and nonlethal severe *P. vivax* malaria, network interactions are found between TNF and parasitemia, HO-1 and SOD-1 [217]. Furthermore, heme induces MP shedding from uninfected RBCs *in vitro*, a process that is inhibited by N-acetylcysteine. This suggests a heme-mediated oxidative stress pathway for the generation of RBC-MPs [218]. Free Hb may also scavenge nitric oxide (NO) and cause low NO-bioavailability, which contributes to the pathogenesis of experimental CM [219]. This is further discussed in section 4.1.3.

3.2.2. Hemozoin

Heme is released during Hb degradation in the food vacuole of the parasite. The released heme generates oxidative stress in the cell and is thus toxic for the parasite. As a detoxification process, heme molecules are dimerized and used as building blocks for stacking into a crystal known as malaria pigment or Hz (reviewed in [220, 221]). Upon schizont rupture, Hz is released into the extracellular environment as a remnant body, which may still contain the membrane of the food vacuole. In the circulation Hz interacts with plasma components and several cell types, including ECs, RBCs and phagocytes, before it is rapidly removed by phagocytosis (reviewed in [222, 223]). These processes may trigger the activation, upregulation and/or downregulation of specific inflammatory reactions [74, 220, 221, 224]. For example, complement deposition with subsequent recognition by CRs and interaction between Hz-bound fibrinogen and TLR4 mediate phagocytosis [52, 74]. The initial contact of Hz with phagocytes can trigger ROS production. In the cell Hz can activate the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome resulting in IL-1β production [225, 226], nuclear factor kappa B (NF-kB) signaling to induce the expression of various proinflammatory cytokines, chemokines, and activation/maturation receptors including TNF, monocyte chemoattractant protein-1 (MCP-1/CCL2), and CD83, whereas contradictory data exist for TLR9 (reviewed in [221, 223]). Furthermore, purified Hz can induce BAFF production by monocytes *in vitro* and thereby modulate B cell subset maturation and survival as discussed in section 2.2.3 [197]. Some of these effects are induced by the core crystal itself, whereas others are induced by lipoperoxidation products associated with Hz such as hydroxyeicosatetraenoic acids (HETEs) and 4-hydroxynonenal (4-HNE) formed by lipid peroxidation of polyunsaturated fatty acids present in membranes.

Depending on the amount of Hz produced, Hz induces inflammation (section 4.2.3) or immune dysfunction (section 5.4) due to intoxication of phagocytic cells [227, 228]. Local Hz-induced inflammation strongly correlates with lung and liver pathology in mice [229, 230], and highest Hz deposition is observed in brain, placenta and bone marrow from severe malaria patients, indicating that Hz may also be pathogenic in human malaria infections [231-233]. Furthermore, Hz cannot be degraded and remains inside the body for a long time [229, 234, 235]. Whether ‘stored’ Hz can still influence phagocyte functions after the infection is cleared is not known.

3.2.3. Glycosylphosphatidylinositol anchors

GPI glycolipids are conserved among all eukaryotic cells and are involved in anchoring membrane proteins to the plasma membrane. GPIs are abundantly present on *Plasmodium* parasites, among others on

692 the sporozoite and merozoite surface coats and in the schizont cytoplasm, and can be distinguished from
693 host cell GPIs by their substituents on the lipid and carbohydrate moieties [236]. GPI-anchors are
694 recognized as endotoxins by the host and induce proinflammatory responses through interaction with
695 TLR2 (and to a lesser extend with TLR4) in a myeloid differentiation primary response gene 88
696 (MyD88)-dependent way [51, 237]. GPIs can also be recognized by CD1d-restricted NKT cells and
697 vaccination against malarial GPI protects mice from severe disease [238, 239]. Furthermore, GPIs induce
698 the expression of tissue factor (TF) on ECs, which activates the coagulation cascade (see also section
699 4.2.2) [240]. GPI-anchors are also suggested to play a role in severe anemia, since insertion of synthetic
700 GPIs into the membrane of noninfected RBCs formed a complex *in vitro* that was recognized by anti-GPI
701 IgGs and IgMs of a patient [241].

702 **3.2.4. Uric acid precipitates**

703 Uric acid is generated from hypoxanthine during nucleic acid metabolism. The parasite cannot synthesize
704 purines *de novo* and acquires hypoxanthine from the host through specialized transporters [242].
705 Although the enzyme required for conversion of hypoxanthine into uric acid is not present in the
706 *Plasmodium* parasite, uric acid precipitates do accumulate within the parasitophorous vacuole of *P.*
707 *falciparum,* mainly in mid- and late-stage parasites, and these precipitates are released in the circulation
708 upon schizont rupture [242, 243]. Furthermore, hypoxanthine may also accumulate in iRBCs and when
709 large amounts of hypoxanthine are released in the circulation, it may be converted into uric acid and
710 further into monosodium urate (MSU) crystals [221]. Uric acid is both protective and detrimental,
711 depending on the cellular localization. In plasma, uric acid scavenges radicals and protects cells from
712 oxidative damage [244]. *In vitro*, uric acid, together with ROS released during the conversion of
713 (hypo)xanthine, stimulates pyrogenic cytokine secretion, e.g. TNF and IL-6, by murine DCs and human
714 PBMCs, and induces maturation of human DCs by increasing costimulatory expression [243, 245, 246].
715 Uric acid precipitates also promote DC expansion and $CD8^+$ T cell activation by triggering the release of
716 Flt3L by mast cells [159]. Furthermore, due to its highly inflammatory effects, uric acid and its
717 precipitates may contribute to severe malaria. In *P. falciparum*-infected Malian children, plasma uric acid
718 levels increase with disease severity and are positively correlated with markers of inflammation and
719 endothelial activation [247, 248]. A pathogenic role of uric acid was also suggested for malaria-associated
720 kidney damage, fever and splenomegaly [244, 247, 249].

721 **3.2.5. Nucleosomes**

722 Together with Hz, GPIs, heme and uric acid, nucleosomes (histone-DNA complexes) are released into the
723 extracellular environment by the rupturing schizont. *Plasmodium*-derived nucleosomes are

724 immunologically active, since they induce DC maturation and cytokine production in a TLR9-dependent
725 way [180, 250, 251]. The cell types expressing TLR9 differ between mice and men, and, therefore, both
726 species may respond differently to plasmodial DNA [252]. In humans, only pDCs and B cells express
727 TLR9, whereas in mice TLR9 is also expressed by mDCs and macrophages. It is important to note that in
728 mice, the effects of TLR9 are dependent on the genetic background, and experiments in TLR9-deficient
729 mice with an insufficiently defined background result in confounding phenotypes [253]. We therefore
730 advocate that in all such studies the genetic background and the numbers of backcrosses should be
731 described in detail.

732 Remarkably, *P. falciparum* histones can also activate microvascular ECs *in vitro*, resulting in a Scr-p38
733 MAPK-dependent induction of IL-8 and other inflammatory mediators, and in endothelial barrier
734 dysfunction by disruption of junctional proteins [250]. The latter feature might be associated with severe
735 complications, since increased permeability and disruption of the BBB and the alveolar-capillary
736 membrane has been shown in CM and malaria-associated acute respiratory distress syndrome (MA-
737 ARDS), respectively (see section 4.2.4). In addition, circulating human and *Plasmodium* nucleosomes are
738 associated with disease severity in *P. falciparum*-infected patients, suggesting a pathogenic role for both
739 host and parasite-derived histones [250].

740 **3.2.6. Microparticles and exosomes**

741 MPs and exosomes are extracellular vesicles that are involved in intercellular communication (reviewed
742 in [254-256]). They differ in their size, formation and composition. MPs are larger than exosomes and are
743 directly shedded from the plasma membrane through vesiculation during cell activation or during other
744 processes including senescence and apoptosis. Consequently, MPs are rich in phospholipids and
745 membrane surface proteins and may carry parasite molecules when produced by iRBCs. MPs derived
746 from iRBCs are more potent in activating macrophages than iRBCs, an effect mediated by TLR4 and
747 MyD88-signalling [257]. These circulating MPs are highly associated with CM in both humans and mice
748 [258-260]. MPs produced by other cell types including ECs, leukocytes and platelets, are also implicated
749 in malaria pathology but will be discussed later (see section 4.2.3).

750 Exosomes are smaller than MPs and are of endocytic origin. They are released after fusion of
751 multivesicular bodies (MVBs) with the plasma membrane for the disposal of cellular material, for
752 example during the maturation process of reticulocytes. Therefore, exosomes are enriched in intracellular
753 components such as proteins of the endosomal pathway and microRNAs (reviewed in [261, 262]). iRBCs
754 also produce exosomes, which are derived from Maurer's clefts (MCs), since *Pf*PTP2, a MC residing

protein, is necessary for MC vesicle budding and intercellular communication [263]. *P. falciparum*-iRBCs use exosomes for intercellular communication as demonstrated *in vitro* by the transfer of drug-resistance plasmids. This exosome-mediated intercellular communication between *P. falciparum*-iRBCs efficiently increased differentiation into gametocytes and may thus be important for transmission to mosquitos [263, 264]. Furthermore, immunization with purified exosomes isolated from the nonlethal reticulocyte-prone *P. yoelii* 17XNL strain, in combination with CpG, is able to elicit IgGs that recognize *P. yoelii*-iRBCs and that protect these mice from a lethal infection when challenged with the normocyte-prone 17XL strain of *P. yoelii* by altering cell tropism to reticulocytes [265].

3.3. Immune evasion mechanisms

Besides the various factors released during the asexual blood-stage that can modulate the immune response, *Plasmodium* parasites have developed mechanisms to actively evade antimalarial immunity, including cytoadherance and antigenic variation and diversity as will be discussed below.

3.3.1. Cytoadherence

Cytoadherence is a major immune evasion mechanism and is mediated by variant surface antigens (VSAs). The presence of VSAs on the surface of the iRBCs allows them to interact with a wide range of receptors on the microvascular endothelium, uninfected RBCs and platelets, and results in endothelial cytoadherence, rosette formation and platelet-mediated clumping, respectively.

3.3.1.1. Endothelial cytoadherence and spleen remodeling

In the spleen, a small percentage of blood flows into the open spaces of Billroth, which is devoid of endothelial lining and which contains a reticular network of fibers and cells together with highly active macrophages [36]. This slows down the blood flow and facilitates interaction between iRBCs, resident phagocytes and other patrolling immune cells. When entering the venous circulation, cells need to squeeze through the interendothelial slits of the sinuses, which traps iRBCs displaying a decreased deformability. These two mechanisms render the open circulation of the spleen highly capable of removing parasites.

Endothelial cytoadherence allows iRBCs to sequester in the microvasculature of multiple organs and to evade passage through the spleen. *P. falciparum*-iRBCs sequester by interacting with different adhesion receptors, including CD36, ICAM-1, chondroitin sulphate A (CSA), heparin sulphate (HS), and endothelial protein C receptor (EPCR) [8, 266, 267]. Which receptor is used by the parasite depends on the receptor availability in the vascular bed and the VSAs expressed on the iRBC surface. Interestingly,

some of the receptors utilized for cytoadherence to e.g. ECs are the same as those used by the immune system for parasite recognition by phagocytes e.g. CD36 (see section 2.1.1) [268]. Microvascular sequestration of iRBCs is a generalized feature of *P. falciparum* malaria and occurs mainly through *Pf*EMP-1. The antigenic variation of *Pf*EMP-1 and the pathological consequences of *Pf*EMP-1-mediated cytoadhesion are discussed later in this manuscript (see sections 3.2.2 and 4.1). *Pf*EMP-1 molecules are encoded by the *var* multigene family and are presented on iRBC membrane protrusions, also known as knobs, which are formed by intracellular knob-associated histidine rich proteins (KAHRP) (reviewed in [267]). These proteins associate with cytoskeletal proteins under the erythrocyte membrane such as actin, spectrin, and ankyrin, and reduce membrane deformability. This stiffness is further increased by the insertion of other parasitic proteins in the *P. falciparum*-iRBC membrane, such as subtelomeric variable open reading frame proteins (STEVORs), members of the *Plasmodium* interspersed repeat (*pir*) multigene superfamily. The stiffness is important to retain the cytoadherent iRBC firmly attached under flow conditions [269-271]. Both *Pf*EMP-1 and KAHRP have no homologues in other *Plasmodium* species. In *P. knowlesi*, schizont-infected cell agglutination (SICA) antigens encoded by the *SICAvar* multigene family are expressed on the iRBC membrane. A proteomic study identified common sequences with *Pf*EMP-1, suggesting that both protein families might have a common ancestor [272].

Sequestration of other human and murine malaria parasites is less well documented. *P. vivax*-iRBCs are thought to sequester less because all stages can be observed in the peripheral circulation, they lack *Pf*EMP-1 orthologs and sticky knob structures, and *P. vivax* infection increases the deformability of iRBCs. The latter is (partly) attributed to the multiple invaginations observed on the infected red cell surface, i.e. caveola vesicle complexes containing *P. vivax*-encoded antigens [2, 202, 273]. Due to this increased deformability, *P. vivax*-iRBCs pass easily through the interendothelial slits of the sinuses, thereby avoiding splenic entrapment. Nevertheless, as with *P. falciparum*, *P. vivax* total parasite biomass is underestimated by peripheral parasitemia levels and is associated with severe disease [274]. Furthermore, peripheral parasitemia, but not total parasite biomass, is associated with endothelial activation, suggesting that parasites rather sequester in organs devoid of endothelial lining such as the open circulation of the spleen and nonvascular parts of the bone marrow. *P. vivax*-iRBCs are observed in spleen biopsies taken from splenectomized patients with acute *P. vivax* infections [275, 276], and possibly also on lung autopsies from deceased *P. vivax* patients from the Brazilian Amazon region [275, 277]. Furthermore, *in vitro* experiments demonstrated cytoadherence of asexual *P. vivax* isolates, although to a variable degree, to microvascular ECs derived from human lungs, simian brains, and to placental cryosections, whereas conflicting data are found with human umbilical vein ECs [278-282]. Several host receptors were identified, such as CSA and hyaluronic acid (HA), but not CD36 or thrombospondin.

819 Cytoadherence to ICAM-1 is also shown with *P. vivax* isolates from the Brazilian [278, 281] and
820 Colombia Amazon [280], but not with Thai isolates [279]. VIR proteins, encoded by the *P. vivax*
821 interspersed repeat (*vir*) genes (reviewed in [283]), seem to be (partly) involved. *Vir* genes are members
822 of the *pir* multigene superfamily, which are present in the different *Plasmodium* species, since
823 homologues are found in *P. falciparum* (*rif/stevor*), *P. knowlesi* (*kir*), *P. berghei* (*bir*), *P. chabaudi* (*cir*)
824 and *P. yoelii* (*yir*).

825 Besides immune evasion, adhesion of reticulocyte-restricted parasites may also play another role. During
826 experimental mouse infection with the nonlethal reticulocyte-prone strain of *P. yoelii*, the open circulation
827 temporarily changes into a 'closed' circulation due to the formation of a physical barrier in the cords
828 made out of fibroblasts, and results in trapping of reticulocytes [9]. *P. yoelii* 17XNL-infected
829 reticulocytes were found to cytoadhere to these barrier cells. Similarly, at autopsy of a *P. vivax* patient,
830 large numbers of *P. vivax*-iRBCs were observed in the cords of the spleen. Whether they were simply
831 there because of mechanical trapping or because of active cytoadherence remains to be determined [275,
832 276]. It was suggested that the trapping of newly formed reticulocytes in the spleen by temporally closing
833 the circulation, and the cytoadherence of iRBCs to these barrier cells to immobilize the mature-stage
834 parasites, could create a favorable niche in which merozoites are released into a reticulocyte-rich
835 environment to enhance invasion [36]. Also sequestration in the bone marrow, another reticulocyte-rich
836 environment, might favor invasion [202, 273].

837 Whether *pir* homologues in other *Plasmodium* species (*kir, bir, cir* and *yir*) also mediate cytoadhesion is
838 currently unknown. *P. chabaudi* has been suggested to sequester in lungs and liver, but the parasite
839 proteins involved in this process remain to be identified [284]. The expression of *cir* genes is tissue-
840 specific and changes during the course of infection, indicating that CIR proteins are expressed and could
841 be important for parasite survival [285, 286], but no role for these in cytoadhesion has been shown so far.
842 *P. berghei* ANKA-iRBCs sequester in lungs and adipose tissue in a manner that is partially dependent on
843 CD36 [287], and in the placenta through a yet unknown mechanism [288]. Sequestration in adipose
844 tissue, and to a lesser extent in the lungs, is mediated by schizont membrane-associated cytoadherence
845 protein (SMAC), an intracellular protein that is not encoded by the *bir* or *Pb-fam-3* multigene families
846 [289]. Until now, no protein is found that is encoded by the *bir* or *Pb-fam-1* multigene superfamily, that is
847 expressed on the outer membrane of the iRBC and that is involved in sequestration [290, 291].
848 Furthermore, *in vitro*, *P. berghei* ANKA was shown to adhere to murine brain and lung microvascular
849 ECs under flow conditions by binding to vascular cell adhesion molecule-1 (VCAM-1), and this type of
850 binding was found to occur less with *P. berghei* K173, a strain that does not cause CM [292].
851 Cytoadherence of the lethal strain of *P. yoelii* was shown to occur in an *ex vivo* cytoadherence assay, and

was mediated by ICAM-1 but not by VCAM-1 [293]. No *P. yoelii* proteins are identified yet involved in this cytoadherence.

3.3.1.2. Rosetting

Rosettes are developed when iRBCs bind to uninfected RBCs. This process may be beneficial for the parasite by shielding epitopes on the iRBC from opsonizing IgGs. Another possible role is to facilitate merozoite invasion by bringing uninfected RBCs into close proximity of the bursting schizont, and to protect against invasion-inhibiting Abs [294]. With *P. falciparum*, both hypotheses seem plausible, and probably depend on the adhesin variant and the host molecule mediating rosetting. Furthermore, rosetting contributes to pathology, since higher rosetting frequencies are observed with *P. falciparum* isolates from African children with severe disease [295, 296]. Rosette formation occurs in post-capillary venules where RBC velocities and shear stress are low, and large aggregates can be found at venular junctions [297]. Since this is also the place where endothelial cytoadherence occurs, both mechanisms contribute to microvascular obstruction.

The mechanisms involved in *P. falciparum* rosette formation with asexual parasite stages are well characterized, and include the interaction between *Pf*EMP-1 and CR1, and the involvement of ABO blood group antigens [8]. *Pf*EMP-1 interaction with group O antigens through binding with the sialic acid (SA) moiety of glycophorin A (CD235a) results in the formation of small rosettes, whereas larger rosettes are formed in the presence of type A RBC by binding of *P. falciparum*-encoded repetitive interspersed families of polypeptides (RIFINs) with group A antigens on A-type RBC [298]. *In vitro*, *P. falciparum* also seems to have a preference for A-type RBCs to form aggregates. STEVORs are also involved in rosette formation and interact with glycophorin C (CD236R) [299]. This latter interaction is proposed to promote invasion by protecting against invasion-inhibiting Abs. Pentameric IgM appears to strengthen the binding between iRBCs and uninfected RBCs in rosettes and is able to bind nonspecifically with its Fc region to two molecules of the *Pf*EMP-1 HB3VAR06 variant [300]. This binding, however, does not protect the iRBC from opsonized phagocytosis. Nonspecific binding of IgM is also reported with another *Pf*EMP-1 variant, VAR2CSA, which mediates binding to the syncytiotrophoblast surface through CSA. This interaction does block the recognition of the iRBC by protective IgGs without affecting cytoadhesion to CSA [301]. Also other serum components such as complement factor H, albumin and self-Abs against band 3 possess rosette-promoting properties [75]. Rosetting is also reported with other parasite species, including the human parasite *P. vivax* [281, 282, 299] and the rodent parasite *P. chabaudi* [302]. Rosette formation with *P. vivax* is more common than with *P. falciparum*, and is mediated by glycophorin C, but not by glycophorin A or CR1 [299]. The hypothesis that rosetting through glycophorin C binding

facilitates merozoite invasion seems very unlikely for *P. vivax*, since mature erythrocytes rather than reticulocytes form rosettes and *P. vivax* almost exclusively infect reticulocytes.

3.3.1.3. Platelet-mediated autoagglutination

Due to their high adhesive phenotype, iRBCs also interact with each other to form autoagglutinates or clumps, a process mediated by platelets (reviewed in [8]). Platelets act as bridging cells between two iRBCs and autoagglutination only occurs with those *P. falciparum* lines that are able to bind to platelet-CD36, whereas contradictory data exist for globular C1q receptor (gC1qR/HABP1/p32) and P-selectin (CD62P) [303-306]. Platelet-mediated autoagglutination is associated with CM, since 100% of *P. falciparum* isolates from Thai patients with CM induced clumping compared to 40-41% of isolates from uncomplicated or severe non-CM patients [307]. Furthermore, autoagglutinates formed by CM isolates were much bigger than those formed by isolates from patients without CM, suggesting that they may cause microcirculatory obstruction and impair blood flow in the brain. Platelets may also act as bridges between iRBCs and ECs and, as such, mediate endothelial cytoadhesion in the microvasculature of organs devoid of specific receptors such as the brain that normally does not express CD36 [308]. Autoagglutination has also been found in blood samples of *P. vivax* patients, but the mechanism of this has not been described yet [309].

3.3.2. Antigen variation and antigen diversity

Protective Abs are generated by the immune system and interfere with invasion and cytoadherence as described in section 2.1.3. In order to establish a prolonged chronic infection that allows continuous parasite transmission, many pathogens, including *Plasmodium* species, evade the immune response by altering the expression of surface proteins, a process called phenotypic or antigenic variation (reviewed in [31, 310]). Furthermore, antigenic diversity, i.e. small clonal variations in the sequence of exposed proteins, enables the parasites to evade immune mechanisms elicited to the same antigen during previous exposures. Both mechanisms contribute to the failure of the host to generate protective immunity, and are a major bottleneck for the generation of an effective vaccine [311].

The mechanisms of antigenic variation are best characterized for the *var* multigene family encoding *Pf*EMP-1, the main parasite ligand involved in cytoadherence. On average 60 *var* genes are distributed in clusters across the different chromosomes. They are divided into three main subgroups, group A, B and C *var* genes and two intermediate groups B/A and B/C, in addition to the more conserved genes *var1*, *var2csa* and *var3*. These subgroups differ in their genomic localization, i.e. subtelomeric or close to the centromeres, and in their extracellular domain structure [312, 313]. Furthermore, a set of conserved

tandem domain cassettes have been identified [314]. Some of these are found in all *P. falciparum* genomes and code for a domain structure that spans the entire length of the *Pf*EMP-1 protein, such as *var1* (domain cassette 1), *var2csa* (domain cassette 2), and *var3* (domain cassette 3), whereas the remaining domain cassettes encode two to four domains. The association between specific domain cassettes and severe malaria is discussed later in section 4.1.1.

Expression of the *var* gene family is subject to allelic exclusion, meaning that one parasite expresses only one variant at a time, and switching to another variant occurs to evade specific Ab-responses [31]. Unlike African trypanosomes which comprise more than thousand variant surface glycoprotein *(vsg)* genes, the *P. falciparum* genome contains only ~60 members of the *var* gene multifamily (even though the overall *var* gene repertoire is thought to be extremely large due to the extensive diversity of this multigene family between strains and isolates). How the parasite prevents rapid exhaustion of the repertoire during chronic infection is not known, but different scenarios are proposed [31]. For instance, parasites may adjust the antigen switching rate to the time necessary for the host to mount a protective immune response or they may use a kind of sensing machinery, which would change switching rates depending on the presence of Abs at the surface of the iRBCs. Which variant is expressed at the surface of the iRBC is tightly regulated to ensure that only a single *var* gene is expressed at a time while the rest of the family is maintained transcriptionally silent. The mechanisms regulating this on-off switching have not yet been elucidated completely, but it is known that it is a highly organized process that mainly relies on epigenetic processes including post-translational histone modifications and differential sub-nuclear localization [31, 315, 316]. Recently, it was found that antisense long noncoding RNAs (lncRNAs) initiating from *var* introns are incorporated into chromatin, and that expression of these antisense lncRNAs in *trans* triggers activation of a silent *var* gene [317]. Also the expression of specific *var* gene subgroups is tightly regulated, as *Pf*RNase II, a chromatin-associated exoribonuclease, mediates the degradation of nascent RNA and controls the silencing of group A *var* genes implicated in the pathogenesis of CM [318]. Furthermore, the overall organization of *var* genes is conserved among genetically different parasites, but their repertoires vary.

It is interesting to note that a high rate of recombinations in the first exon of the *var* genes has been detected during mitosis of the erythrocytic stage of the parasite [319]. These recombinations preserve the domain structure of the *Pf*EMP-1 protein and may result in the formation of large numbers of new antigenic structures within a single patient, suggesting that the *var* gene sequence polymorphisms arise during the asexual parts of the life cycle of the parasite.

Also other surface-exposed epitopes on iRBCs, e.g. RIFINs and STEVORS and other members of the PIR multigene family, in the different *Plasmodium* species are subject to antigenic variation. For example, multiple RIFIN and STEVOR variants may be expressed simultaneously onto the iRBC surface [31, 320, 321], and antigenic variation of RIFIN and STEVOR proteins on the merozoite surface may contribute to immune evasion by camouflaging conserved merozoite surface antigens involved in the invasion process [31]. Furthermore, vector transmission of *P. chabaudi* attenuates parasite virulence through modulation of the *cir* gene expression and by inducing an enhanced immune response [322]. Whether both phenomena are interrelated is currently unknown.

Antigenic diversity is important in the multigene families and also in other genes encoding for immunologically exposed proteins, in particular proteins on the surface of merozoites and proteins involved in erythrocyte invasion. This diversity is maintained in parasite populations by balancing evolution (see section 1.1) and also contributes to immune evasion. This became particularly clear in the trials with vaccine candidates such as MSP proteins and AMA-1, which were able to generate efficient immune responses against parasites carrying the same allele of the antigen, but the field efficacy remained low because of the low cross-reaction to diverse allelic variants present in the population [323, 324].

4. Imbalances result in severe pathology

A perfectly tuned balance between antiparasite immunity and immune evasion by the parasite is crucial to avoid pathology (Figure 2A). Most infected individuals manage to fulfill this demanding task, often supported by antimalarial treatment. However, in approximately 1% of the infected patients, mostly nonimmune residents, travelers and primigravidae, this balance is disrupted and severe disease ensues. Severe complications are most common with *P. falciparum*, but also with *P. vivax* and *P. knowlesi*, severe disease with a possible lethal outcome is increasingly reported [325-327].

Therefore, we will discuss how imbalances can exacerbate the development of pathologies and may be life-threatening or even lethal. Extensive blood vessel obstruction might occur when the generated immune response is insufficient to limit parasite replication (section 4.1). Thereby, a highly activated endothelium supports widespread cytoadhesion of mature-stage iRBCs in the microvasculature of several organs. Conversely, an exaggerated inflammation may occur, with equally devastating consequences (section 4.2). Both situations are compared in Figure 5, and may occur separately or simultaneously in specific organs in either patients or mouse models, leading to distinct complications which are classified according to the immunological imbalances in Figure 2 and section 6.

4.1. Blood flow obstruction causes hypoxia

Parasite sequestration may cause metabolic disturbances by compromising the blood circulation and thus by inducing hypoxia and lactic acidosis. This may occur in several tissues and organs, including the brain, the lungs and the placenta, and can further be aggravated by local inflammation and immunopathological reactions that damage vascular integrity (Figure 5A). These imbalances are central in a variety of complications, including CM, placenta-associated malaria (PAM), MA-ARDS and acidosis [328].

4.1.1. Excessive iRBC sequestration

In *P. falciparum*-infections, the extent of iRBC sequestration varies between individual microvessels, between tissues and according to the severity of the infection [329]. Although it also occurs in patients with uncomplicated or asymptomatic infections, high organ-specific parasite loads are associated with severe disease as demonstrated in brains and lungs of patients who died from CM or MA-ARDS and in placentas from women with *P. falciparum*-associated PAM [233, 329-333]. Furthermore, in CM high parasite loads are also found in several other organs besides the brain, including lungs, intestine, kidneys and skin, indicating that CM is characterized by a high total parasite biomass [334-336]. The parasite load in *P. vivax*-infections is much lower than with *P. falciparum*, but with both parasites total parasite biomass is associated with severe disease [274].

Antigenic variation promotes effective sequestration and thus contributes to pathology. In people infected with *P. falciparum*, some *var* members are dominantly expressed or confer a growth advantage within specific tissues and are associated with severe complications. As an example, positive selection of infected red cells expressing VAR2CSA (domain cassette 2) occurs in primigravid women due to the presence of the placenta where they can bind to CSA [337, 338]. Sequestration of iRBCs (and subsequently phagocytes) at the maternal side of the placenta reduces the blood flow through the intervillous area and results in thickening of the trophoblastic basement membrane and impaired transport of maternal nutrients and oxygen across the placenta to the fetus [339]. Interference with this binding by specific Abs prevents PAM, highlighting the importance of site-specific sequestration in malaria pathology. For other complications, the association between particular *var* gene expression and pathology is less clear. A clinicopathological study performed on fatal pediatric patients in Malawi suggested that distinct *var* genes are dominantly expressed in the different organs of the same patients, although further conclusions are difficult in view of the technical limitations [340]. *Var* genes containing domain cassettes 8 or 13 are preferentially upregulated in iRBCs that bind to primary microvascular ECs from brain, lungs and skin, are associated with severe malaria and are recognized by Abs from young African children from malaria-endemic regions [108, 341-343]. Interestingly, *Pf*EMP1 variants containing domain cassettes 8 or 13 bind to EPCR, which is important in the regulation of inflammation and coagulation, and whose expression is decreased in the brain of CM patients [344, 345]. These data indicate that severe *falciparum*

malaria is associated with a small subset of *var* genes that promote a particular type of microvascular sequestration, conferring a growth advantage in malaria-naïve individuals. Besides cytoadherence to the endothelium, rosetting and autoagglutination, if present, also contribute to microvascular obstruction and were found to be associated with severe disease in African children [295, 296, 346].

Differences in the cytoadherence pattern exist between human and rodent parasite species and strains, which may in part be due to the species-specific nature of these adhesin-receptor interactions. In both *P. falciparum*-infected patients and mice with severe malaria infections, heterogeneous obstruction of microcirculatory blood flow was recorded, and the degree of obstruction was proportional to the extent of ischemia and to the disease severity [347-354]. In *P. falciparum*-infected patients with CM, massive iRBC cytoadherence is observed in the brain microvasculature [329, 335]. The intravascular presence of leukocytes in the brain has long been debated, however, an increasing number of recent papers clearly show the presence of monocytes and platelets on brain autopsies from fatal CM patients alongside with iRBCs [355, 356]. In mice with experimental CM, brain microvascular obstruction is mainly mediated by cytoadherent $CD8^+$ T cells and $ICAM1^+$ monocytes/macrophages in the presence of accumulating iRBCs [357-361]. Interestingly, *P. berghei* ANKA parasites were found to be slowed down in the cerebral microcirculation, but they never became completely arrested [362]. Regardless of differences in the cell type causing the obstruction, obstruction with associated hypoxia is present, as witnessed by the heterogeneous pattern of neuronal and perivascular hypoxia that is found scattered throughout the brain and that is significantly higher in brains of mice with CM compared to non-CM brains [363].

Both iRBCs and inflammatory cells are present in the pulmonary microvasculature of mice and patients with MA-ARDS or CM [277, 287, 289, 364-366]. Also in placentas of mice and *P. falciparum*-infected patients, high numbers of iRBCs and leukocytes accumulate in the intervillous maternal space and adhere to CSA at the syncytiotrophoblast surface rather than directly to the microvascular endothelial lining [367-370]. The expression of variants binding CSA creates a favorable niche for optimal parasite replication since monocytes/macrophages are not well equipped to phagocytose CSA-binding iRBCs as these do not bind to CD36 (see section 2.1.1) [371]. This may lead to high parasite burdens in the placenta, and parasites may be undetectable in the peripheral circulation while high parasite loads (>30% iRBCs) are locally found in the placenta [333].

Severe disease affecting brain, lungs and placenta is increasingly reported with *P. vivax* [325-327]. It is currently unclear whether local *P. vivax* sequestration occurs and significantly contributes to these types of severe pathologies [277, 372]. Sequestration is suggested to occur in spleen and bone marrow, where it contributes to systemic inflammation. Thus, a different underlying disease mechanism seems to be

operating in severe *P. vivax* disease, in which systemic and local inflammation rather than local microvascular obstruction due to iRBC sequestration seem to play a dominant role [274].

Hypoxia-inducible factor-1α (HIF-1α), a transcription factor specifically upregulated by hypoxia, is similarly in*duce*d in brain ECs of mice with and without CM. Activation of HIF-1α triggers the transcription of several genes implicated in protection against ischemia, i.e. genes encoding proteins involved in glycolysis, blood flow and oxygen delivery to tissues including vascular endothelial growth factor (VEGF) and erythropoietin (Epo) [373]. For example, in the brain, Epo protects neural tissues against oxidative damage, among others by stimulating neural stem cell proliferation and differentiation and neuron survival [374]. Whereas HIF-1α could not be detected in *postmortem* brains of severe malaria patients, HIF-2α, VEGF and Epo staining was detected at similar levels in different areas of CM and non-CM brains [375, 376]. Epo receptor (EpoR) staining is significantly higher in CM brains than in non-CM brains, and the frequency of vascular HIF-2α and EpoR staining is positively correlated with iRBC sequestration, indicating that they are principally upregulated in local hypoxic areas [375, 376]. In mice with CM, administration of exogenous Epo decreased cerebral hypoxia, HIF-1α, VEGF and activities of calpain and caspase and improved survival [377]. Whether exogenous Epo is also beneficial as adjunctive treatment for human CM is currently under investigation. Even though high plasma Epo levels are associated with protection against neurological sequelae in Kenyan children, warranty is needed since high plasma Epo in Ugandan children is associated with prolonged coma duration and increased mortality [378, 379]. In general, these data indicate that local hypoxic areas exist in the brain that are associated with sequestration, but that hypoxia itself is not sufficient to explain cerebral pathology.
Hypoxia may also be induced in malaria-infected placentas, since *hif-1α*, *cox-1* and *cox-2*, but not *vegf* mRNA expression are observed in placentas from Colombian patients with submicroscopical infections [380].
Hypoxia induced by local decreases in blood flow is further aggravated by impaired oxygen delivery, e.g. caused by anemia or hypovolemia [381]. Increased oxidative stress induced by blood parasites may oxidize Hb into metHb, which is unable to bind oxygen. Increased metHb and carboxyHb levels have been documented in uncomplicated and severe malaria infections [382, 383] and in mice with experimental CM [219], and may contribute to impaired oxygen carriage and delivery. Furthermore, oxygen consumption in tissues, as measured in skeletal muscles from patients with uncomplicated and severe malaria, is increased and further contributes to the imbalance between oxygen delivery *versus* oxygen demand [384, 385].

4.1.2. Metabolic changes

Hypoxia results in increased lactate concentrations as is observed in venous blood and cerebrospinal fluid (CSF) from mice and patients with severe complications, and hyperlactatemia is strongly associated with a fatal outcome [350, 386-390]. Lactate, together with other unidentified anions, lowers the pH of the blood and acidemia develops when the natural buffering capacity of the blood is exceeded, further complicating severe disease [391]. Respiratory distress in African children with severe malarial anemia (SMA) often reflects lactic acidosis due to hypoxia, since blood transfusion (i.e. increasing oxygen supply) decreased blood lactate levels and improved respiratory abnormalities [392]. Lactate could possibly also have a damaging effect on brain endothelium during CM [393]. Hyperlactatemia may have different origins, including production by the parasite itself and by uncontrolled muscle contraction during seizures [387, 394]. Nonetheless, the most important contributor is anaerobic respiration in poorly perfused tissues as suggested by increased lactate:pyruvate ratios in patients with complicated malaria [395], the association of lactate levels with poor microvascular function (defined as the capacity to increase oxygen delivery in response to ischemia), both in severe *P. falciparum* and severe *P. vivax* infections [274, 385], and the association between lactate and total parasite biomass and blood vessel obstruction [353]. Increased blood lactate levels can also be explained by decreased lactate clearance. However, the fact that hepatic gluconeogenesis, which is the major route for lactate clearance, is not impaired argues against this hypothesis [387, 396]. Furthermore, lactate acidosis does not appear to originate from hypovolemia and should therefore not be treated with liberal fluid resuscitation, as this may result in pulmonary edema [397].

Bicarbonate is the major extracellular buffering system in the body, because it can be adjusted very effectively by lungs and kidneys. Thus, when blood pH decreases, the body will respond by expelling more carbon dioxide (CO_2) by increasing the breathing rate or depth. Deep breathing is frequently observed in patients with complicated malaria and is often a sign of severe metabolic acidosis, a complication which is independently associated with mortality according to one of the largest randomized control trials with more than 5000 African children with severe *falciparum* malaria [398]. Furthermore, bicarbonate levels are decreased in severe malaria compared to uncomplicated malaria [399], and in patients who died from CM or survived with complications compared to patients who made a full recovery [400].

Hypo- and hyperglycemia (reviewed in [401]) and other metabolic disturbances also occur in malaria, but are out of the scope of this review.

4.1.3. Altered vasomotor activity

To optimize oxygen delivery and to limit tissue toxicity in hypoxic areas, local or systemic compensation mechanisms are activated. Most tissues respond to hypoxia by vasodilation. However, if this occurs in the brain, intracranial pressure increases resulting in brain swelling and decreased cerebral perfusion pressure. Brain compression together with decreased perfusion may further aggravate the already existing hypoxia as observed in unconscious children and adults with CM [402-404]. Furthermore, when intracranial pressure is severely increased, brain stem compression and cardiorespiratory arrest may ensue which often results in death or neurologic sequelae upon survival. Besides vasodilatation, edema is another mechanism leading to increased intracranial pressure and is discussed in section 4.2.4. Since brain swelling appears to be a crucial determinant for the outcome of cerebral malaria, it may also imply that ventilatory support, to maintain the patient breathing till the exaggerated intracranial pressure resolves, might be life-saving [405].

Based on the literature, it is hard to conclude if compensatory vasodilation occurs or not. Increased blood flows have been observed in microvessels adjacent to clogged ones in brains of CM patients and in larger vessels in rectal mucosa of severe malaria patients [348, 406]. In mice with hyperparasitemia and anemia, cerebral blood flow was increased and minor brain swelling was observed [407]. Furthermore, several mediators are produced during infection that can regulate the vascular tone by influencing vascular smooth muscle cell contraction. Endothelin-1 (ET-1) is a potent vasoconstrictor and is produced by injured or ischemic ECs and in response to TNF. Bioactive ET-1 levels and its precursor big-ET-1 are increased in malaria patients [408, 409], and ET-1 is significantly induced at the transcriptional level in brains of mice with CM [410]. A novel inhibitor of ET-1 provided some protection against cerebral malaria in mice when administered as adjunctive therapy in combination with artemether, corroborating the pathogenic role of ET-1 [411]. *In vitro* studies indicate that even though endothelial ET-1 secretion is increased during hypoxic situations, iRBCs are able to scavenge ET-1 and thereby inhibit ET-1 functions [412]. Since substantial iRBC sequestration occurs in the microvasculature, this may be a strategy by which the parasite prevents vasoconstriction. Also angiotensin II (AT-II) promotes vasoconstriction, however, genetic polymorphisms in angiotensin I converting enzyme (ACE-I) or ACE-II, which result in increased levels of AT-II, are associated with protection against CM [413]. The latter finding may be related to the antiplasmodial activity of AT-II [414]. Interestingly, inhibition of either ACE or the AT_1-receptor with captopril or losartan protects against murine CM by decreasing splenic T cell activation [415].

NO is one of the main vasodilators and decreased NO bioavailability may be an important mechanism by which compensatory vasodilation is impaired. Low NO bioavailability is observed in a large proportion of patients with severe complications and in mice with CM and may have different etiologies [219, 384, 416,

417]. Due to intravascular hemolysis, large amounts of free Hb and arginase are released in the circulation. Free Hb may scavenge NO produced by ECs resulting in MetHb and nitrate formation, whereas arginase will convert L-arginine into L-ornithine. In this way, less L-arginine is available for NOS-mediated production of NO. Furthermore, when L-arginine concentrations are low, NOS will preferentially synthesize superoxide radicals (O_2^-) which may interact with NO to form the highly reactive peroxynitrite ($ONOO^-$). The decrease in tetrahydrobiopterin, an important cofactor for NOS-mediated NO production, and the concomitant increase in dihydrobiopterin further favors the peroxynitrite formation and oxidative stress at the expense of NO production [418]. Indoleamine-2,3-dioxygenase (Ido)-derived kynurenine is produced by the inflamed endothelium and contributes to arterial vessel relaxation and hypotension in *P. berghei*-infected mice with and without CM [419]. Furthermore, plasma levels of C-type natriuretic peptide (CNP), another vasorelaxant mediator, are slightly decreased in severe malaria patients [408].

In general, the local vasomotor tone will depend on the overall balance between mediators inducing vasoconstriction or vasodilation. Accordingly, the vascular tone may be differentially regulated in large and small vessels and between different vascular beds. Consequently, measuring systemic levels of these mediators may not be informative to predict the local microvascular tone. Thus, even though kynurenine is induced by the vascular endothelium, vasoconstrictors probably predominate during CM in mice, since hypoargininemia, increased MetHb levels and microcirculatory collapse are observed and pharmacological vascular smooth muscle cell relaxation by exogenous NO, CO, iloprost (a synthetic prostacyclin analog) or nimodipine (a calcium channel blocker) improved survival chances from CM [213, 219, 350, 351, 420, 421]. Inhaled NO also resulted in decreased systemic inflammation and endothelial activation in murine CM [422]. Interestingly, exogenous NO-administration also protects against murine CM through an unrelated mechanism. It activates the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf-2), which is a sensor of oxidative stress [423]. Nrf-2 subsequently induces HO-1, which produces CO that protects by binding to cell-free Hb, blocking its oxidation to toxic and inflammatory MetHb and other derivatives. This protective mechanism of NO is also related to the decreased activation of pathogenic T cells. Furthermore, phenoxybenzamine, an alpha-blocker and vasodilator, and exogenous CO prevented pulmonary edema in *P. berghei*-infected mice [424, 425]. Together these data indicate that cerebral and pulmonary complications in mice are most likely associated with impaired vasodilation. In human malaria patients, however, the occurrence of vasodilation may be a more heterogeneous phenomenon with variations in patients, organs and vessels.

4.2. Disproportional activation of proinflammatory immune mechanisms

A strong and robust immune response is required to combat the infection. However, the activated cells and the produced mediators will also interact with the surrounding vessel walls thereby promoting a procoagulant state and increased permeability. When inflammation and endothelial dysfunction exceed a treshold, edema and/or hemorrhages may occur leading to parenchymal tissue damage and ultimately organ dysfunction, as is observed in several malarial complications, including MA-ARDS.

4.2.1. Excessive endothelial activation

Systemic microvascular endothelial activation occurs due to the combined effects of sequestration, inflammation and the locally released iRBC content during schizont rupture, including heme, Hz, histones and GPIs (see section 3.2) [240, 250, 426-430]. This results in exocytosis of Weibel-Palade bodies (WPBs), secretion of inflammatory mediators, such as cytokines, chemokines and proteases, tissue factor (TF) expression and in upregulation of adhesion molecules. Thus, by measuring plasma levels of mediators that are normally stored inside WPBs, e.g. angiopoietin-2 (ANG2) and von Willebrand factor (vWF), or soluble adhesion molecules like sP-selectin and sICAM-1, the extent of EC activation can be determined.

EC activation is observed in asymptomatic and symptomatic malaria infections and in infections of other etiologies [431, 432]. In human volunteers experimentally infected with *P. falciparum*, vWF levels increase immediately after the onset of blood-stage infection, indicating that endothelial activation is one of the first alterations induced by infection [433]. Furthermore, higher levels of endothelial activation markers, including ANG2, sICAM-1, and sE-selectin are found with *P. vivax* compared with *P. falciparum*, despite of significantly lower parasite counts, indicating that *P. vivax* is a more potent activator of the endothelium [434].

The extent of EC activation is much higher during pathology. ANG2, ANG2/ANG1 and vWF levels increase with disease severity and are associated with parasite biomass, lactate levels and mortality rates [432, 435-439]. *Postmortem* analyses of CM brains show high ANG1, ANG2 and Tie2 neuronal and endothelial staining [439]. Neuronal ANG1 and ANG2 staining are associated with microhemorrhages, whereas no association is found with parasite sequestration. ICAM-1 is also increased in the cerebral endothelium of CM patients [440-442], and may augment parasite sequestration [443]. Also in *P. vivax* infections markers of endothelial activation are increased and correlate with severity and with peripheral parasitemia [444-446]. No correlation is found with parasite biomass, indicating that circulating iRBCs rather than the hidden component activate the endothelium.

The ANG-Tie2 axis is able to rapidly modify the endothelial response to possible pathogenic insults such as infection and inflammation [447]. ANG1 is constitutively produced and promotes endothelial quiescence by signaling through the Tie2 receptor, whereas ANG2 promotes vascular permeability and endothelial activation by blocking this receptor. NO regulates this axis by inhibiting the release of ANG2 from WPBs and by inducing ANG1 [448]. As already mentioned, in a large proportion of patients with severe complications and in mice with CM, NO bioavailability is decreased (see section 4.1.3). Thus, due to the combined effects of sequestration, inflammation and dysregulation of endothelial activation, pro-inflammatory signaling cascades are activated, which may lead to endothelial secretion of pro-inflammatory mediators, alteration of vasomotor activity, cytoskeletal rearrangements resulting in permeability changes, induction of a procoagulant state and/or induction of EC apoptosis [433, 449-451].

4.2.2. Coagulation changes

Coagulopathy is common in severe malaria and results from a combination of several factors, including thrombocytopenia and activation of the coagulation pathway [452]. It is indicative of a poor prognosis and may manifest itself as diffuse intravascular coagulation (DIC), ring-form hemorrhages in the brain during CM (thereby defining the CM2 subgroup of patients), microhemorrhages in lungs or other forms of bleedings [452, 453]. A generalized procoagulant state results in consumption and exhaustion of platelets and coagulation factors, which, combined with endothelial damage, results in an increased sensitivity to hemorrhages.

Thrombocytopenia is induced rapidly after the onset of a blood-stage infection [433] and is frequently observed in *P. falciparum* and *P. vivax* patients [305, 454-459] and in mouse models of malaria [460-462]. A strong correlation is found between thrombocytopenia and levels of both vWF and activated vWF [433]. Endothelial vWF is stored as ultra-large (UL)-vWF multimers or as vWF propeptide in WPB. When released during EC activation, these UL-vWF multimers unravel under flow and form platelet-decorated strings which may bind large numbers of iRBCs [451]. In normal situations, these prothrombogenic multimers are rapidly cleaved by the protease a disintegrin and metalloproteinase with a thrombospondin type I motif member 13 (ADAMTS13). However, *P. falciparum* and *P. vivax* patients with severe malaria infections have significantly reduced ADAMTS13-activity [274, 399, 454, 463]. Thus, the combination of endothelial activation with increased circulating amounts of active and UL-vWF, together with reduced vWF inactivation by ADAMTS13, may result in intravascular platelet aggregation, as observed in brains and lungs of severely ill patients and mice [308, 356, 461, 464-466] and in *P. falciparum*-infected placentas [308]. In *P. vivax* patients, platelet numbers are also positively correlated with ANG1 and negatively with ANG2 and the ANG2/ANG1 ratio [444].

Furthermore, the urokinase plasminogen activating receptor (uPAR) may participate in endothelial platelet adhesion [467]. Platelet depletion or inhibition of platelet adhesion to the endothelium, e.g. by uPAR deficiency or treatment with aprotinin (a nonspecific inhibitor of plasmin that also interferes with platelet adhesion to the endothelium), significantly decreases mortality from pulmonary and cerebral pathology and ameliorates thrombocytopenia in mice, underlining the pathological role of platelet sequestration in brain and lungs [461, 464, 468-470].

Platelet adhesion to ECs provides additional receptors to microvascular beds that otherwise lack important adhesion receptors, e.g. CD36 in the brain, and may, therefore, enhance iRBC sequestration to the activated endothelium [308]. Increased iRBC sequestration together with platelet-mediated clumping augments immune evasion and favors parasite replication, which may contribute to higher parasite loads and aggravate blood-flow obstruction and microvascular disease. Furthermore, platelet adhesion potentiates the cytotoxic effects of iRBCs on the activated endothelium, including EC apoptosis. They further increase permeability and reduce transendothelial electrical resistance (TEER) [449] and they promote inflammation by secretion of inflammatory or proapoptotic molecules upon activation, e.g. transforming growth factor-β1 (TGF-β1) and PF4/CXCL4 [470, 471]. TGF-β1 induces EC apoptosis and PF4/CXCL4 contributes to leukocyte recruitment, T cell activation and cytokine production by monocytes. Thus, even though platelets are implicated in antimalarial immunity, they also significantly contribute to severe malarial disease.

In addition to platelet trapping, increased platelet turnover and active platelet removal may also contribute to low platelet numbers in human malaria [145, 457]. Both IgG-mediated platelet phagocytosis and RNIs are negatively correlated with platelet counts. Decreased levels of circulating platelets are also associated with an increase in the number of MPs, both in mice and in patients, and inhibition of caspase-mediated apoptosis of platelets decreases the number of MPs and increases platelet numbers [462, 472]. Bone marrow megakaryopoiesis and thrombopoietin production to replenish peripheral platelet numbers seems to be normal or slightly increased, indicating that thrombocytopenia originates mainly in the periphery. Thus, intravascular platelet adhesion, altered platelet kinetics, active platelet removal and increased platelet-derived MP formation may all contribute to thrombocytopenia. Furthermore, thrombocytopenia is also associated with peripheral parasitemia levels [305, 455, 458].

The coagulation cascade is also activated during severe malaria as evidenced by the upregulation of TF in human and mouse CM brains [450, 473]. Mature-stage iRBCs induce TF expression on dermal microvascular ECs *in vitro* resulting in the assembly of the TF/factor VIIa (FVIIa) complex, which cleaves and activates coagulation factor X. The latter is crucial in the activation of thrombin, which

converts fibrinogen into fibrin. Furthermore, MPs promote a procoagulant state, since they may express significant amounts of TF, and because they contain large amounts of surface-exposed PS [474]. This recruits vitamin K-dependent and gamma-carboxyglutamyl residue-containing coagulation factors (FVII, FIX, FX and FII), which significantly enhances the coagulation cascade.

Levels of anti-thrombin-III (AT-III) are decreased [475, 476] and thrombin-anti-thrombin (TAT) complexes are increased [475, 477] in plasma of severe malaria patients, indicating that thrombin formation occurs during malaria infection and that coagulation inhibitors are consumed. Plasminogen activator inhibitor-1 (PAI-1) levels are increased in malaria patients and levels of fibrin degradation products (FDPs) seem to increase with disease severity [478]. Also protein S, protein C and EPCR are decreased, resulting in a lower inhibition of the coagulation cascade [345, 478]. Furthermore, in *P. vivax*-infected patients thrombocytopenia and coagulation seem to be interrelated, since a negative correlation is found between platelet counts, TAT complexes and D-dimers (reviewed in [145]).

4.2.3. Inflammation

Inflammation is associated with malaria. Whether this inflammation is mild or excessive and contributes to pathology depends on the interplay between parasite and host. Insufficient or ineffective immune responses result in high parasite load and the release of inflammation-regulating components by the parasites (Figure 2A). Excessive inflammation, even at moderate parasitemia, is at the basis of severe immunopathological complications in several murine malaria models (Figure 5B). The role of inflammation in SMA is discussed in section 4.2.5. Also during MA-ARDS and PAM in both mice and patients, abundant leukocyte recruitment and inflammation are evident. Since MA-ARDS often appears after therapeutic clearance of parasites, it is supposed to result from excessive inflammation. In CM patients iRBC sequestration clearly dominates inflammatory infiltrates. Although signs of inflammation are present, it is currently unclear and heavily debated to what extent inflammation contributes to CM pathology, among others because anti-inflammatory therapy of CM patients failed to improve survival [479]. Little or no iRBC sequestration is present in peripheral organs of severe *P. vivax* patients and inflammatory processes at low parasitemia prevail. In this section, we review the different processes which initiate, sustain and aggravate localized inflammatory reactions and associated damage in malaria.

4.2.3.1. Initiation of inflammation

It is currently unclear which events initiate inflammation. In static *in vitro* cocultures, late-stage parasites activate inflammatory pathways in ECs, e.g. through activation of NF-kB, resulting in the production of cytokines, chemokines and adhesion molecules [427, 480]. Although the need for cytoadherence in this

process has not been formally proven, it is reasonable to assume that parasite sequestration results in local endothelial inflammation by the localized release of inflammatory compounds such as free heme, Hz, GPI anchors, nucleosomes, and uric acid precipitates and precursors. These immunomodulatory molecules are highly inflammatory as previously discussed (see section 3.2) and could be associated with severe complications due to excessive inflammation. Also the interaction between parasite adhesins and adhesion molecules on ECs may trigger activation of signaling pathways [481].

4.2.3.2. Activated phagocytic cells

Both monocyte/macrophage and granulocyte populations expand during malaria infection to augment the parasite clearance capacity. High organ-specific parasite loads are associated with severe disease [233, 274, 329-333], and the proportion of circulating phagocytes containing Hz increases with disease severity [482-484]. Phagocytes clean up the iRBC content released during schizont rupture and thus prevent these components to cause damage to the vessel wall. Intravascular marginating and/or infiltrating phagocytes with or without ingested Hz are observed in brains, lungs and placenta of both mice [367, 485-493] and patients [333, 355, 356, 364, 371, 494-500] with CM, malaria-associated acute lung injury (MA-ALI)/ARDS or PAM, respectively. Furthermore, tissue macrophages e.g. microglia, Kupffer cells and alveolar macrophages, are activated during infection in mice and humans [347, 491, 497, 501-503]. Widespread microglial activation is not limited to areas of petechial bleedings or iRBC sequestration and precedes accumulation of leukocytes and *in situ* proliferation of $CD8^+$ T cells [491, 503]. Both in human and mouse placentas, high numbers of monocytes/macrophages accumulate in the intervillous space, especially during chronic infection [332, 333, 367, 371, 495, 500], and high levels of placental chemokine expression are associated with monocyte infiltration in human and mouse placentas [367, 504]. Local phagocytosis of iRBCs occurs mainly in *P. falciparum*-infected placentas as evidenced by the presence of Hz, parasites and RBCs (both infected and uninfected) in various stages of degradation [332]. Nevertheless, phagocytes fail to remove the majority of iRBCs, and this reduced phagocytic capacity may partly be related to the inability of VAR2CSA-expressing iRBCs to bind to CD36 (see also sections 2.1.1 and 4.1.1) [371]. Because of this impaired nonopsonic phagocytic response, more monocytes/macrophages are recruited to the placenta and high amounts of toxic mediators, cytokines and chemokines are produced. This further augments inflammation and contributes to pathology, with partial syncytiotrophoblastic necrosis as a common finding in placentas with high numbers of accumulating macrophages [332].

4.2.3.3. Cytotoxic T cells

$CD8^+$ T cells are also observed in the placental intervillous spaces of both infected and non-infected women, and CTL numbers are higher in women with chronic *P. falciparum*-infections, especially in those with massive intervillous inflammation [495]. Furthermore, the number of intervillous CTLs are related to the percentage of maternal iRBCs and the extent of Hz deposition [495]. Intervillous CTLs from infected women are primed to produce IFN-γ and TNF after *in vitro* mitogen stimulation [505], indicating that proinflammatory immune responses are induced in the placenta. In murine CM, more than 90% of brain sequestered CTLs present express the CXC chemokine receptor 3 (CXCR3) [506], which is crucial for the cerebral pathogenesis in mice [470, 488, 507, 508]. NK cell-derived IFN-γ and PF4/CXCL4 stimulate expression of CXCR3 on activated T cells in the spleen [470, 506], and IFN-γ also induces Interferon gamma-induced protein 10 (IP-10)/CXCL10 expression in the brain, resulting in recruitment of activated T cells to the cerebral vasculature [488, 507, 509]. IP-10/CXCL10 is also associated with human CM, as highest IP-10/CXCL10 CSF levels are detected in CM patients, plasma IP-10/CXCL10 levels increase with disease severity and increased levels are tightly associated with CM mortality [510, 511]. In mouse malaria models both parasite-specific and nonspecific CTLs are generated, although mainly the parasite-specific CTLs induced by cross-presentation of blood-stage malarial antigens by the $CD8\alpha^+$ subset of mDCs in the context of MHC class I are pathogenic [512, 513]. Several antigens may be recognized by these activated $CD8^+$ T cells, including glideosome-associated protein, and this can occur in murine models both with and without CM [514, 515]. The DCs driving the activation of these cells also express $Clec9A^+$ [516]. Clec9A is a C-type lectin-like receptor expressed by murine $CD8\alpha^+$ mDC and pDCs and also by human DCs [517]. This subset of mDCs also expresses BDCA3 and is also augmented in malaria patients [518]. Flt3L derived from mast cells is responsible for the expansion of these $CD8^+$ DCs, and their depletion abrogates *P. berghei* ANKA-induced CM in mice [159].

In addition to the migration of the activated $CD8^+$ T cells to the brain, a crucial event is the acquisition and cross-presentation of malarial antigens by brain ECs. Upon sequestration of *P. falciparum*-iRBCs onto ECs, the ECs appear to acquire malarial antigens, and may become targets for adaptive immunity [519]. Also platelets can acquire and cross-present *Plasmodium* antigens in association with MHC class I molecules with the necessary costimulatory signals and contribute to the generation and *in situ* proliferation of parasite-specific CTLs [190, 520]. $CD8^+$ CTLs compromise the BBB by releasing perforin and granzyme and by inducing apoptosis through Fas-FasL interaction with subsequent leakage of protein-rich fluid across the BBB [358, 521-524]. In CM patients, increased intracranial pressure is a major finding and might be due to edema. However, the precise cause of the brain swelling has not yet been determined (see following section) and $CD8^+$ T cell accumulation has not been reported. $CD8^+$ T cells may have a more prominent pathological role in human MA-ARDS, since both in mice and in

humans the alveolar-capillary membrane is damaged with the presence of mononuclear cell infiltrations and extensive interstitial and/or alveolar edema formation [364, 365, 425, 489, 490, 499, 525], and depletion of $CD8^+$ T cells or IP-10/CXCL10 deficiency in MA-ARDS-susceptible mice prevents pulmonary edema [428, 489, 526, 527]. Furthermore, CTLs contribute to spleen remodeling by killing metallophilic macrophages in the inner border of the marginal zone [528], which could compromise the generation of an optimal splenic immune response (see section 5.5).

4.2.3.4. Cytokines and other soluble inflammatory mediators

Due to the presence of these inflammatory cells in peripheral organs, considerable amounts of cytokines (e.g. TNF, IL-1β, IL-6), ROS and other inflammatory mediators [e.g. gelatinase B/matrix metalloproteinase-9 (MMP-9) and VEGF] are produced that further enhance inflammation, participate in endothelial remodeling, and contribute to tissue injury. For example, high amounts of ROS produced by phagocytic cells can cause oxidative damage. In *P. vivax*, mainly $CD14^{hi}CD16/Fc\gamma RIIIA^+$ 'inflammatory' monocytes produce high amounts of ROS [45]. Antioxidative treatment protects mice from CM [529], even though no increased levels of protein carbonylation nor oxidation are found in the brain, and mice deficient in NADPH oxidase are as susceptible as WT to develop CM [66]. The antioxidative defense in the brain of mice with CM appears to be mediated by HO-1 and glutathione peroxidase, whereas the expression of SOD and catalase is decreased [530]. Antioxidative adjunctive therapeutic treatment of severely ill *P. falciparum* patients was not effective [531].

Also excessive cytokine production may further enhance pathology. Local inflammatory reactions are best characterized in the murine CM model. Already early in the infection IFN-γ-producing NK cells are among the first cells to be recruited to the brain in murine CM [506]. This increases the expression of adhesion molecules and chemokines by the cerebral endothelium and further enhances leukocyte recruitment and sequestration [488, 532]. The adhesion molecules P-selectin, ICAM-1, and its ligand LFA-1 support leukocyte and platelet adhesion [461, 464, 493, 533-535], and chemokines, including CC-chemokines [e.g. MCP-1/CCL2 and macrophage inflammatory protein-1α (MIP-1α/CCL3)] and CXC-chemokines [e.g. PF4/CXCL4, monokine induced by gamma-interferon (MIG/CXCL9), IP-10/CXCL10 and interferon-inducible T-cell alpha chemoattractant (I-TAC/CXCL11)] recruit more leukocytes to the brain, including activated $CD4^+$ and $CD8^+$ T cells [488, 507, 508, 536]. The importance of IFN-γ in murine CM is further illustrated by the fact that depletion or deficiency of IFN-γ or the IFN-γ receptor (IFN-γR) protects mice from CM [488, 537-542] and IFN-γR expression is only upregulated in brains of mice with CM [543]. Excessive IFN-γ production is also associated with cerebral pathology in human disease, since highest IFN-γ staining is detected in brains of *P. falciparum*-patients who died from this

neuropathology [544], and both IFN-γ and IFN-γ/IL-10 ratios increase with disease severity in *P. vivax*-patients [545]. Inflammation, as shown by increased peripheral blood levels of IFN-γ, IL-2 and TNF, is also high during placental malaria in pregnant women submicroscopically infected with *P. vivax* [380]. Most peculiarly, T cell trafficking to the lungs and pulmonary chemokine expression in mice are increased in the absence of IFN-γ signaling [546, 547], indicating that different mechanisms orchestrate brain and pulmonary inflammation.

An important determinant of IFN-γ expression and immunopathology in mice is the NK complex (NKC) [548, 549]. The NKC is a genomic region that is comprised of different genes and multigene families encoding receptors that positively or negatively influence the cytotoxic activity and/or cytokine production by cells expressing these receptors, i.e. NK cells and NKT cells. The susceptibility of mouse strains towards malaria immunopathology is in part determined by the NKC genotype, since introduction of the NKC of disease-susceptible mouse strains (e.g. C57BL/6) into disease-resistant strains (e.g. BALB/c) increases the susceptibility to severe pathology. Among the cell types expressing these NKC-encoded receptors, mainly CD1d-restricted NKT cells control systemic proinflammatory cytokine production, because in both CD1d-deficient BALB/c mice and BALB/c mice containing the C57BL/6-NKC, IFN-γ responses were not timely downregulated and no switch towards IL-4 production was observed [548]. Two specific loci in the C57BL/6 are responsible for this effect [550]. Thus, by their cytokine secretion, CD1d-restricted NKT cells determine the magnitude of the proinflammatory response and, as such, influence the individual outcome in the balance between antiparasite immunity and inflammation.

Also cytokines of the TNF family play important roles in murine CM. Interaction of lymphocyte-derived lymphotoxin α_3 ($LT\alpha_3$)/$LT\alpha_1\beta_2$ with TNF receptor 2 (TNFR2) and LTβ receptor (LTβR) activates the endothelium and augments monocyte/macrophage, T cell and platelet adhesion in the brain by increasing ICAM-1 expression on microvascular ECs [449, 551, 552]. Moreover, several cytokines synergize to augment the expression of adhesion molecules [429]. Furthermore, TNF can alter the local vasomotor tone by inducing the release of vasoconstrictors by the endothelium, e.g. ET-1 [408], and can induce MP shedding from the endothelium [449]. TNF levels increase with disease severity and are maximal in severe malaria patients, both with *P. falciparum* and *P. vivax* [446, 477, 545, 553-562]. Highest brain levels of TNF are observed in *P. falciparum*-patients who died from cerebral pathology [442, 544]. Despite the pathogenic role of TNF in malaria pathology, deficiency of TNF or TNFR1 has no effect on murine CM [538, 539, 551, 563-567]. Furthermore, neutralization of TNF in patients with CM appeared effective to suppress fever, but it did not improve survival and rather increased neurological sequelae

[568]. This might be due to the neutralization of the protective actions of TNF, since TNF is important for antiparasite immunity [569-571], or due to the actions of other cytokines such as IFN-γ and LT-α that also contribute to cerebral pathology (see above).

4.2.3.5. Microparticles

MP shedding may further augment inflammation. Higher levels of circulating MPs of different origin, including from iRBCs (see section 3.2.6), noninfected RBCs, ECs, and platelets, are found in severe *P. falciparum* malaria [258, 572] and murine models of CM [260]. MPs are also produced during acute *P. vivax* infection, but the amounts are lower compared to uncomplicated *P. falciparum* infections [218, 472]. Whether increased MP shedding is also associated with severe *P. vivax* disease remains to be investigated. Inflammation contributes to MP production, since TNF induces a higher degree of MP shedding from ECs derived from aspirated subcutaneous tissue of patients with CM than from ECs from patients with uncomplicated malarial disease [573], and circulating MPs of endothelial origin are highest in malaria patients with cerebral pathology [258]. Furthermore, MPs activate diverse immune mechanisms *in vitro*, e.g. iRBC-derived MPs are potent inducers of macrophage activation as discussed in section 3.2.6 [257], and platelet-derived MPs bind to iRBCs, are internalized by human brain microvascular ECs and transfer platelet antigens such as CD36 to the iRBC and EC surface [574]. Interestingly, endothelium-derived MPs may express MHC class II and other molecules important for antigen presentation and are able to enhance $CD4^+$ and $CD8^+$ T cell activation [575]. MPs also contribute to immunopathology, as adoptive transfer of labelled plasma MPs from mice with CM indicates that MPs rapidly interact with ECs and induce neurological and pulmonary insults [576]. Furthermore, inhibition of MP formation, e.g. by genetic targeting of the ATP-binding cassette transporter A1 (ABCA1) or by treatment with the low-molecular-weight thiol panthetine, protects mice from CM [260, 577]. Despite of this, MPs are not invariably harmful, as they may protect the endothelium and prevent blood loss [474, 578].

4.2.4. Vascular hyperpermeability

Malaria infections may compromise the endothelial barrier in specific organs. Extensive non-cardiogenic pulmonary edema is well established in both mice and patients with MA-ALI and in its more severe form MA-ARDS (reviewed in [453]). VEGF may increase vascular permeability by dissociation of vascular endothelial (VE)-cadherin from its cytoskeletal anchoring protein β-catenin [579] and by redistribution of actin, occludin and zona occludens-1 (ZO-1) [580]. VEGF is positively correlated with edema in lungs of mice with MA-ARDS [229], and contributes significantly to fatal edema in mice with MA-ALI [425]. In normal situations, pulmonary VEGF is predominantly produced by epithelial cells and diffuses through

the alveolar-capillary membrane where it binds to VEGF receptors on the vascular endothelium [581]. However, during MA-ARDS VEGF transcription is downregulated probably due to Hz-induced inflammation that damages the alveolar epithelium [229]. Thus, VEGF-mediated pathology probably arises due to high numbers of VEGF-containing immune cells that are mobilized from the spleen to the lungs, rather than from *de novo* synthesis of VEGF in the lungs [229, 425]. Sequestration of iRBCs occurs in the pulmonary microvasculature in mice [287, 582, 583] and in *P. falciparum*-infected patients [334, 499], and it is still questionable with *P. vivax* [277, 364]. In an isolated perfused lung system, sequestering iRBC were shown to produce ROS, which decreases TEER indicating barrier loss [584]. Furthermore, parasite components released during schizont rupture, e.g. merozoites, GPIs and food vacuoles, can alter junctional morphology and disrupt ZO-1, claudin 5 and VE-cadherin in primary lung microvascular ECs resulting in interendothelial gap formation [585]. Hz, another component released by rupturing schizonts, induces inflammation in the lungs, and Hz levels and pathology are strongly correlated with the expression of several inflammatory mediators and the number of immune cells in the lungs, including activated $CD8^+$ T cells [229], which contribute to pathology probably by disrupting junctional integrity [489]. Alveolar fluid clearance mechanisms are also impaired and contribute to the increase in vascular permeability. TNF, which is induced in the lungs by e.g. Hz, is positively correlated with pulmonary pathology [229], and reduces the expression and activity of the amiloride-sensitive epithelium sodium channel (ENaC) involved in alveolar fluid clearance [581]. Both ENaC expression and activity are decreased in lungs of mice with malaria-associated edema [490].

The occurrence of pathological vascular permeability changes in CM is less clear. Massive vasogenic and also cytotoxic edema are observed in murine CM brains [350, 428, 563, 586, 587], and strategies to prevent vascular hyperpermeability protect mice against CM [588]. The bidirectional water channel aquaporin 4 (AQP4) is an important regulator of water movement between fluid compartments (blood and CSF) and brain parenchyma, and facilitates water movement into brain astrocytes in cytotoxic edema and water movement out of the brain in vasogenic edema [589]. In murine CM, deficiency in AQP4 aggravates the neuropathology [587]. Inflammation and vasogenic edema in murine malaria are tightly linked. The hyperpermeability may be directly caused by endothelial activation (see section 4.2.1). As ECs may also acquire malarial antigens (see section 4.2.3), it is also tempting to speculate that $CD8^+$ T cell cytotoxic activity towards ECs may be a main cause of endothelial barrier damage.
Focal loss of ZO-1, occludin and vinculin between ECs constituting the BBB is only observed in human CM [590] and *in vitro* studies showed that only iRBCs from CM patients were able to down-regulate the transcription of these junctional proteins [591]. However, diffuse microscopic cerebral edema, including enlarged perivascular spaces, AQP4 upregulation and VEGF signaling without significantly increased

brain weight are frequently observed in adult severe malaria patients with and without CM, and no correlations are found with neuropathology [592]. Furthermore, mannitol adjunctive treatment, which decreases intracranial pressure by removing water from the brain parenchyma, does not improve survival from CM [404, 593]. With neuroimaging of carefully diagnosed CM patients, increased intracranial pressure is one of the most common findings [403, 404, 594-596], and in a recent large magnetic resonance imaging (MRI) study, severe brain swelling was found in 84% of lethal CM cases (with retinopathy), whereas in nonlethal cases the brain swelling was transient [405]. However, the exact etiology is currently unclear and might involve venous congestion and/or vasogenic and cytotoxic edema [597]. Differences between Asian adults and African children may also be involved. The availability of improved MRI technology in endemic areas will hopefully clarify this issue [598].

4.2.5. Erythrocyte destruction and ineffective erythropoiesis

Hematocrit levels depend on a homeostatic balance between RBC destruction and *de novo* synthesis in hematopoietic tissues. During malaria, RBC destruction is enhanced and, if compensating erythropoiesis is impaired, this leads to anemia (reviewed in [599, 600]). Mild to moderate anemia develops in many *P. falciparum* and *P. vivax*-infected patients, and in endemic regions malaria is one of the most prevalent causes of anemia. A small percentage of all infected individuals, mainly young children and pregnant women, are prone to develop life-threatening anemia, the most common severe complication of malaria infections [326, 327].

Several mechanisms may contribute to increased red cell destruction. Parasite replication results in hemolysis because mature schizonts rupture when releasing the newly formed merozoites. Nevertheless, mainly destruction of uninfected RBCs contributes to anemia, a process that is even more pronounced with *P. vivax* [599]. RBC surface expression of complement regulatory proteins CR1 and CD55 is decreased, while C3b deposition is increased in *P. falciparum*-infected patients with SMA [601, 602]. Increased complement deposition could result from oxidative damage with subsequent band 3 receptor clustering induced by ROS release from phagocytes and/or by transfer of 4-HNE from iRBCs to uninfected RBCs in rosettes [73, 601, 603, 604]. Also direct contact with digestive vacuoles covered with C3 and C5 convertases mediates deposition of activated C3b and C5b components onto the red cell surface [90]. Furthermore, higher surface deposition of IgG and IgM, directed either against autologous RBC surface proteins or against parasite antigens inserted in the noninfected RBC membrane after their release from the parasite, e.g. parasitic GPIs, is observed [241, 605]. Both mechanisms shorten the lifespan of RBCs by targeting these prematurely for CR1-mediated hemophagocytosis. Apoptosis of noninfected RBCs, with subsequent deposition of CRP and phagocytosis mediated by FcγRIIA may

1529 additionally contribute to the development of anemia with *P. falciparum* and *P. yoelii*, although this does
1530 not seem to occur with *P. vivax* [85, 606, 607]. Different mechanisms may dominate in murine models,
1531 since anemia associated with rodent malaria seems to be C3-independent [605]. Activation of the adaptive
1532 immune system may further exacerbate RBC destruction, as $CD4^+$ T cells may contribute by enhancing
1533 phagocyte activation [608], and even $CD8^+$ T cell-dependent parasite clearance may trigger the loss of
1534 uninfected erythrocytes in the spleen through an as yet unknown mechanism [137].

1535 Anemia results in oxygen shortage [609]. This is sensed in the kidney by decreased hydroxylation and
1536 enhanced stability of HIFs, and stimulates Epo production. By promoting the proliferation, differentiation
1537 and maturation of erythroid progenitors, Epo induces erythropoiesis to increase the oxygen delivery
1538 capacity. In pediatric malaria patients, circulating Epo levels increase with disease severity, highest levels
1539 are found in severely anemic patients, and erythropoiesis is impaired (reviewed in [599, 600]). This
1540 suggests that in children the erythropoietic system might be unresponsive to Epo. In contrast, in a large
1541 percentage of infected adults, the ability to produce adequate Epo levels is impaired, which implies that
1542 different mechanisms may be responsible for the inefficient erythropoiesis observed in malaria-infected
1543 children and adults. In *P. chabaudi* AS-infected A/J mice, sufficient numbers of $EpoR^+$ early erythroid
1544 progenitors are present in the spleen, but fail to differentiate and mature properly into Hb-producing
1545 erythroblasts in response to Epo. Consequently, inappropriately low numbers of reticulocytes are released
1546 in the circulation [610]. Impaired differentiation of early erythroid progenitors into erythroblasts has also
1547 been suggested to contribute to anemia in Gambian children [611]. The exact mechanisms driving this
1548 decreased sensitivity towards Epo are not yet fully elucidated, but the release of cytokines (e.g. TNF) and
1549 Hz may be involved. Increased levels of Hz-containing monocytes are associated with anemia and
1550 reticulocyte suppression, and are also abundantly present in organs with erythropoietic activity such as
1551 bone marrow and spleen [612, 613]. Furthermore, Hz-fed monocytes and the Hz-derived product 4-HNE
1552 can inhibit erythroid progenitor growth *in vitro* by interfering with the cell cycle and with the expression
1553 of crucial receptors, e.g. EpoR, the transferrin receptor CD71, stem cell factor receptor, and IL-3R [614-
1554 616]. The inhibitory effects on erythropoiesis mediated by parasite products like Hz are different from
1555 and additional to changes induced by host inflammatory molecules e.g. TNF [617]. Since Hz also induces
1556 the expression of inflammatory molecules, these inflammation-independent effects augment the
1557 importance of malaria pigment in impairing erythropoiesis. Also *P. vivax*-iRBCs are able to inhibit
1558 erythroid development *in vitro*, an effect most probably mediated by the released parasite components
1559 from the rupturing schizont as no TNF nor IFN-γ were detected in the culture medium [618]. The
1560 importance of parasite components in the induction of anemia due to *P. vivax* is further evidenced by the

fact that only parasitemia was an independent predictor of Hb levels, and not the level of inflammation [619].

Hepcidin, the iron regulatory hormone, is upregulated during asymptomatic and uncomplicated malaria and was suggested to contribute to malaria-associated anemia by limiting iron availability for incorporation into erythroid precursors [620]. However, no association was found between hepcidin and Hb levels and low hepcidin levels are found in SMA patients [621-624]. Anti-Epo auto-Abs are negatively correlated with Hb levels in semi-immune mice, which could point to an additional mechanism why erythroid progenitors are not responding accurately [625]. Also phagocytosis of erythroid precursors and dyserythropoiesis, i.e. morphologic abnormalities of the nuclei and cytoplasm, are observed in the bone marrow of malaria patients and contribute to the inadequate erythropoietic response [600]. Together, these data indicate that the interplay between the immune system and parasite-derived virulence factors such as Hz play major roles in the pathogenesis of severe malarial anemia.

5. Immunoregulatory mechanisms dampen antimalarial immunity and immunopathology

As outlined above, an exaggerated proinflammatory response may result in tissue damage. Therefore, different mechanisms are activated in the host to downregulate the proinflammatory response. However, when this anti-inflammatory response is induced too early or when it is too strong, the immune system is not able to control parasite replication. Also the parasite itself can interfere with specific immune mechanisms, and a disorganized architecture of secondary lymphoid organs impairs humoral immunity. Insufficient control of parasite replication may also lead to pathology. In this section, the mechanisms that dampen the immune response, including activation of regulatory T cells, signaling through inhibitory receptors, production of anti-inflammatory cytokines, interference by parasite products and impaired humoral responses are discussed.

5.1. Regulatory T cells

In addition to the activation of Th cells and effector T cells, also T cells with an immunosuppressive phenotype are induced during infection. Regulatory T cells (Tregs) contribute to immune homeostasis by dampening excessive inflammatory responses. How they are induced is not yet completely clarified. Intact *P. falciparum*-iRBCs or soluble extracts can induce the differentiation of immunosuppressive $CD4^{+}CD25^{hi}$ forkhead box P3 transcription factor $(Foxp3)^{hi}$ Tregs *in vitro* [626, 627]. Furthermore, their induction is enhanced by and depends on the synergistic effect of bioactive TGF-β and IL-10 produced by monocytes, is independent of *Pf*EMP-1 surface expression, and IL-2 is required for their proliferation and

activation [626, 628]. One study indicates that TLR9 on DCs is essential for the activation of Tregs in murine malaria with *P. yoelii* 17XL [57].

Tregs are observed during malaria infection in both mice and men [183, 184, 629-634]. In both children and adults with uncomplicated and severe malaria infections, a positive correlation was found between peripheral parasitemia levels and the frequency of $FoxP3^+$ Tregs within the $CD4^+$ T cell pool [184, 635]. Umbilical cord blood from neonates whose mothers suffered from *P. falciparum* placental malaria at delivery contains an expanded population of parasite-specific $CD4^+CD25^{hi}$ and $CD4^+CD25^+$ cytotoxic T lymphocyte antigen 4 $(CTLA\text{-}4)^+$ Treg cells [636]. These cells produce high levels of IL-10 *in vitro* that suppress both effector T cell responses and MHC class I and II expression by monocytes. Also in *P. vivax*-infected individuals, parasitemia correlates with the numbers of $CD4^+CD25^+FoxP3^+$ T cells coexpressing glucocorticoid-induced TNFR (GITR), CTLA-4, IL-10, TGF-β, IFN-γ or IL-17, and these cells inhibit proliferation of PBMCs *in vitro* [184, 634].

In mice infected with the lethal *P. yoelii* 17XL strain, Treg cell depletion with anti-CD25 augments DC activation, promotes clearance of the parasite and results in survival [637-639]. Inducible genetic depletion of $FoxP3^+$ Treg cells also results in enhanced parasitemia control, and this is independent from T cell-derived IL-10 [640]. Similarly, inhibition of the expansion of Tregs with anti-IL-2 during *P. chabaudi* AS infection enhances antiparasite immunity during the chronic phase of infection [641]. With the same parasite strain, transfer of Tregs or induction of Tregs with IL-2/anti-IL-2 complex reduces parasite clearance and enhances IL-10 production [642]. The role of Tregs in *P. berghei*-infections is less clear. In some studies, anti-CD25 treatment decreases parasite burden and protects from experimental CM [643, 644], whereas other studies show limited or no effects of Tregs in CM [631, 633]. These differences might be due to the use of anti-CD25 to deplete Tregs, since CD25 is also transiently expressed on activated $CD4^+$ T cells. Therefore, inducible genetic depletion of $FoxP3^+$ Tregs in DEREG mice may be more reliable as a technique and this did not affect the occurrence of *P. berghei* ANKA-induced CM [645]. Tregs induced with IL-2/anti-IL-2 complex are found to be protective against CM, a mechanism mediated by CTLA-4 [646]. Overall, a major role of Tregs in the pathogenesis of experimental CM has not been found, although technological limitations and also the plasticity and heterogeneity of Treg subsets may be confounding factors. Tregs may have a disease-promoting role in mice and men by suppressing effective antiparasite immune responses and promoting high parasite loads.

Two mechanisms have been described by which Tregs diminish proinflammatory responses [647]. Direct cell-cell contact, e.g. through CTLA-4 or membrane-bound bioactive TGF-β on Tregs, seems to be

essential to attenuate proinflammatory responses in $CD4^+CD25^-$ T cells cocultured with APCs [648]. However, whether this is mediated by direct cell-cell contact between $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells or indirectly through interaction with APCs has not yet been elucidated. Furthermore, Tregs obtained from infected mice and patients secrete high levels of the immunomodulatory cytokines TGF-β and IL-10, which can interact with their corresponding receptors on different cell types [626, 627, 648]. Interestingly, $FoxP3^+$ Tregs are not the major IL-10 producing cells. Highest IL-10 production is by IFN-γ^+FoxP3$^-$ CD4 T cells, as detected in both patients and mouse models [635, 649-651]. In mice, this unusual IL-10 production by Th1 cells is dependent on IL-27 [651]. The latter cytokine plays an anti-inflammatory role in murine malaria, not only by inducing IL-10 in IFN-γ^+FoxP3$^-$ Th1 cells but also by suppressing the IL-12-driven terminal differentiation of Th1 cells [652]. As such, IL-27 plays a dual role as it suppresses efficient parasitemia control in primary and secondary infections, while it also protects against severe inflammatory liver pathology [653, 654], illustrating the importance of this cytokine in steering the immunological balance of malaria toward equilibrium or imbalances. Besides T cells, regulatory B cells also produce IL-10 in murine malaria and may contribute to the suppression of CM [655]. With *P. vivax* also $CD14^{hi}$CD16/FcγRIIIA$^+$ 'inflammatory' monocytes seem to be a source of IL-10 production, which is produced in combination with TNF [45].

5.2. Inhibitory receptors

The proinflammatory response can also be downregulated by signaling through inhibitory receptors expressed by various cells of the immune system. Activated T cells can express inhibitory receptors such as CTLA-4, PD-1 and lymphocyte-activation gene-3 (LAG-3), and signaling through these receptors impairs proinflammatory cytokine production and protective T cell activity [136, 656, 657]. Such $CD4^+$ T cells coexpressing PD-1 and LAG-3 have been found in higher frequencies in children living in regions with persistent *P. falciparum* exposure and were found in combination with high frequencies of atypical MBCs (see section 5.5) [658]. Signalling through these receptors may interfere with the generation of a robust antiparasite response because therapeutic blockade of both PD-1/PD ligand 1 (PD-L1) and LAG-3/MHC class II ligation rapidly improves antiparasitic immunity in several mouse-parasite combinations. Furthermore, perfectly balanced co-inhibitory and co-activation signals are important to prevent excessive proinflammatory cytokine secretion. For example, PD-1 inhibitory signals are important to limit excessive IFN-γ production resulting from OX40 (CD134)-mediated costimulation, since such excessive IFN-γ would otherwise inhibit GC generation important for the production of high affinity parasite-specific MBCs and long-lived PCs as described in section 2.2.3 [194]. When induced at the right time, signaling through the inhibitory receptors CTLA-4 and PD-1 expressed by activated T cells also protects

against immunopathology in mice by dampening excessive proinflammatory cytokine production [656, 659, 660]. Another inhibitory receptor, BTLA is expressed on many leukocytes, binds to herpes virus entry mediator (HVEM) and decreases antimalarial immunity in *P. yoelii* infections through both innate and adaptive immune pathways [661]. In children, highest proportions of CTLA-4 expressing $CD4^+$ T cells are detected in peripheral blood during severe malaria [662], indicating that inappropriate T cell inhibition may prevent the generation of sufficient antiparasite immunity. Furthermore, interaction between IgGs and the inhibitory FcγRIIB on macrophages and activated B cells impairs antiparasite immunity in mice and men [663], and gene polymorphisms abrogating FcγRIIB function are more prevalent in populations living in malaria-endemic areas and are associated with protection against severe disease [664].

5.3. Anti-inflammatory cytokines and enzymes

Inflammation-extinguishing cytokines such as TGF-β are produced by different cell types including Tregs, monocytes/macrophages and platelets, and high plasma levels have been detected early during infection in both nonimmune individuals and in mice infected with lethal malaria parasite strains [629, 665, 666]. TGF-β production is necessary to protect the host against severe pathology by diminishing inflammation [667], as severe *P. vivax* patients have lower TGF-β concentrations compared to patients with mild disease [215]. Decreased TGF-β levels can originate from heme-dependent inhibition of TGF-β secretion by monocytes or by thrombocytopenia, which are both present in malaria. When TGF-β is produced early after infection, it facilitates blood-stage parasite growth by inhibiting protective immune responses. This effect is strongly pronounced in IL-12-deficient mice, in which TGF-β impairs phagocytic responses, and the latter cannot be restored after supplementation with exogenous IFN-γ [668]. TGF-β is constitutively expressed by a wide range of cell types and stored as an inactive, latent protein. Because its activity is not merely regulated at the transcriptional level but also by proteolytic activation, high plasma levels can be obtained rapidly. Mature malaria parasites, both *P. falciparum* and murine malaria parasites, appear to possess a thrombospondin-like molecule and metalloproteinase activity which together can activate latent TGF-β and are thus capable of directly inducing bioactive TGF-β [627, 669]. In this way, the parasite is able to diminish host-derived immune responses thereby supporting parasite survival and transmission. Besides a role in Treg cell activation and function, TGF-β may also directly inhibit proliferation and IFN-γ secretion by NK cells [670]. Whether this occurs in *Plasmodium* infections as observed in other parasitic infections, needs to be investigated. Neutralization of early TGF-β, together with inhibition of the compensatory rise in IL-10, restores early IFN-γ responses and protects mice from a lethal *P. yoelii* infection [665].

IL-10 is also induced during malaria and is observed after the early wave of proinflammatory cytokine secretion [665]. IL-10 alone does not seem to be of major importance for antiparasite immunity in mice, as depletion of IL-10 or supplementation with recombinant IL-10 does not significantly alter peripheral parasitemia, although it diminishes IL-12 secretion by DCs and subsequent IFN-γ responses [104, 665, 671-674]. In patients with uncomplicated *P. falciparum* or *P. vivax* malaria, IL-10 has been implicated in DC apoptosis, as plasma IL-10 levels correlate with circulating apoptotic DCs, and the total numbers of circulating DCs are decreased [185]. Furthermore, IL-10 correlates with total parasite biomass in severe *vivax* patients [274]. In mice, adequate IL-10 production is indispensable to prevent immunopathology, as mice deficient in IL-10 develop CM when infected with *P. chabaudi*, a parasite that is otherwise nonlethal in C57BL/6 mice. Treatment with anti-TNF or anti-IFN-γ Abs rescued these mice from CM, indicating that the protective effects of IL-10 are due to the downregulation of the proinflammatory response [671, 672].

In macrophages, IL-10 inhibits proinflammatory gene transcription e.g. TNF, IL-1β and IL-6 by inducing HO-1 which, by releasing CO, inhibits the translocation of NF-kB to the nucleus (see section 4.2.3) [675, 676]. HO-1 and exogenous CO potently protect mice against severe pathology in different organs including brain, lungs and liver, in particular by reducing activation of pathogenic $CD8^+$ T cells (see also section 4.2.3) [213, 425, 677]. Interestingly, sickle cell Hb in transgenic mice induces HO-1 via the oxidative stress sensor Nrf-2 and protects against CM, suggesting that this might be a mechanism how sickle cell anemia protects against severe pathology in malaria [678]. The same pathway, Nrf-2-mediated induction of HO-1, has been implicated as the protective mechanism of NO (see above, [423]). Both IL-10 and HO-1 protein expression are absent in brains of mice with CM [213, 679]. Why HO-1 is not upregulated in murine CM brains is not known, since free heme, the substrate of HO-1, is liberated from rupturing schizonts (and uninfected RBCs) and should normally induce HO-1 expression. In patients who died from CM, strong HO-1 staining is observed in brain, lungs and liver in intravascular monocytes and tissue macrophages (Kupffer cells and alveolar macrophages) and in monocytes and microglia inside Dürck's granulomas [497, 680, 681]. Furthermore, highest IL-10 levels are found in brains of CM patients [544] indicating that IL-10/HO-1 responses are induced but are not sufficient to protect against CM.

Most peculiarly, both IL-10 and HO-1 are involved in the exacerbation of *Salmonella typhimurium* infections (causes nontyphoid Salmonella, a common and often lethal complication of *P. falciparum* infection in Sub-Saharan Africa) resulting in acute, fatal bacteremia in mice [682]. Granulocytes normally kill these intracellular bacteria by production of ROS. However, since *P. yoelii* 17XNL parasites induce

HO-1 expression in immature bone marrow granulocytes, the maturation of these cells and the generation of an effective oxidative burst is impaired and intracellular bacterial replication is increased. This neutrophil dysfunction and *Salmonella* exacerbation is also dependent on myeloid-cell derived IL-10 [683]. The neutrophil dysfunction also occurs in *P. falciparum* malaria and correlates with hemolysis and HO-1 induction [682]. HO-1 is induced especially abundantly in neutrophils and is highest in patients with severe malaria, and HO-1 mRNA correlated with IL-10 mRNA levels in whole blood [684]. In contrast, neutrophils from *P. vivax* patients are activated and their phagocytic activity and superoxide production is significantly enhanced, although they have a decreased chemotactic activity [685]. Whether also *P. vivax* exacerbates *Salmonella* infections remains to be investigated, however, intracellular killing mechanisms do not seem to be impaired with *P. vivax*.

All together, these data indicate that IL-10 and HO-1 are linked and may provide protection against severe immunopathology, but may also cause impaired immunity, in particular against bacterial infections occurring as comorbidities of malaria.

Type I IFNs have also been implicated in immunomodulation in murine CM, but paradoxical data exist whether type I IFNs are protective or pathogenic [686-688]. Administration of recombinant type I IFN protects against experimental CM by reducing the expression of inflammatory molecules, e.g. TNF, ICAM-1, and MIG/CXCL9 [686, 687]. However, type I IFN signaling hampers DCs to stimulate IFN-γ production by $CD4^+$ T cells and to control parasite growth, indicating that type I IFN also decrease antimalarial immunity, and part of these effects are mediated through interferon regulatory factor 7 (IRF-7) [688-690]. In humans, specific IFN-αR1 polymorphisms are associated with protection against CM [691].

5.4. Parasite products

Besides activation of negative feedback mechanisms to limit excessive inflammation and to protect against pathology, the parasite and its products can also actively interfere with the generated immune response in numerous ways.

For example, Hz may downregulate protective immune responses in multiple ways (besides inducing inflammation and oxidation as discussed in sections 3.2.2, 4.2.4 and 4.2.5). Phagocytosis of Hz generates high amounts of 4-HNE and HETEs that, through activation of and interaction with PPAR-γ, inhibit important monocyte functions *in vitro* involved in phagocytosis and antigen presentation (e.g. by downregulating MHC class II expression) and in differentiating monocytes into DCs [692]. 4-HNE also reacts with the cytoskeleton and impairs chemotaxis of monocytes [693]. PPAR-γ antagonizes NF-kB

signaling by interacting with the p65 subunit of NF-kB, and Hz-derived products such as HETEs can both induce proinflammatory immune responses through activation of NF-kB and downregulate immunity and inflammation by acting on PPAR-γ. Therefore, the eventual immunological effect will depend on the timing of PPAR-γ activation during infection and the amount of trans-inhibition occurring over time in the microenvironment. Interestingly, PPAR-γ agonists enhance parasite clearance and decrease inflammatory responses in patients with uncomplicated malaria [694] by upregulating CD36-mediated phagocytosis of iRBCs [695]. However, under inflammatory conditions PPAR-γ is downregulated and Nrf-2 activators appear more efficient in upregulating CD36-mediated phagocytosis of parasites [696]. Furthermore, since PPAR-γ agonists decrease inflammation they might have beneficial effects in complications due to exaggerated inflammation. In experimental CM, PPAR-γ agonists decrease EC activation and BBB damage and improve survival [697].

Furthermore, in *P. berghei*-infected mice, Hz levels are negatively correlated with MHC class II expression in lungs and livers. In contrast, *P. chabaudi* AS-infections produce much lower amounts of Hz, which do not correlate with MHC class II expression but rather correlate with hepatic inflammation and damage [229, 230]. Anti-Hz IgM Abs have also been detected in serum of patients with complicated *P. falciparum* malaria and can inhibit TNF and IL-1β production by monocytes *in vitro* [698].

The parasite may also interfere with the generation of a proper humoral response. As will be discussed in the following section, *Pf*EMP-1 may trigger T cell-independent polyclonal B cell activation which may interfere with more specific and efficient B cell-mediated immunity.

5.5. Impaired humoral immunity

The generation of Ab-mediated immunity has been discussed in sections 2.1.3 and 2.2.3 and is in part circumvented by the parasite by antigen variation and diversity (see section 3.3.2). Interference in the generation of the antibody response, and in particular in B cell memory, further impedes efficient antimalarial antibody-mediated immunity.

For the induction of an adequate humoral response a properly organized architecture in the secondary lymphoid organs is imperative. However, features of splenic architectural disorganization, e.g. reduced B cell numbers in the marginal zone surrounding B cell follicles (possibly due to apoptosis of MBCs), decreased numbers and/or disorganized GCs, extrafollicular foci of plasmablasts, disappearance of the marginal zone or a fading delineation between GCs and red pulp, have been observed in mice, monkeys and in patients with severe *P. falciparum* disease [699-702], while in patients with uncomplicated

malaria, splenic white pulp hypercellularity and active GC formation are apparent [275, 276]. This disorganization may cause alterations in the proportions of the different B cell populations that are observed.

Furthermore, chronic malaria exposure may promote atypical MBC differentiation over classical MBCs [703]. These atypical MBCs express inhibitory receptors and fail to respond to B cell receptor clustering resulting in impaired B cell responses including proliferation, cytokine production and Ab secretion. [106, 130]. Why and how this occurs is currently unknown, although CD4 T cell exhaustion might contribute to promote atypical MBC generation (see also section 5.2) [658]. Furthermore, whether atypical MBCs differentiate from classical MBCs or whether they originate from different precursors is still controversial [703, 704]. An expanded atypical MBC pool is found in people who are chronically exposed to or infected with malaria, and a higher frequency of atypical MBCs is found in patients who reported at least one previous malaria episode compared to no previous exposure [658, 705-708]. These atypical MBCs upregulate inhibitory receptors such as Fc receptor ligand 3 (FcRL3) and FcRL5, and show diminished BCR signaling, proliferation, and cytokine production [703, 709]. Furthermore, they do not actively secrete Abs (although contradictory data exist) and also do not differentiate into antibody-secreting cells after *in vitro* stimulation as do classical MBCs normally do [703, 704]. Whether atypical MBCs are also present during experimental rodent infections is currently not known. Further studies may indicate whether the expansion of these atypical MBCs is responsible for the inefficient and slow acquisition of antimalarial B cell memory.

Another phenomenon interfering with the generation of efficient and specific antimalarial humoral responses is T cell-independent polyclonal B cell activation, which may cause hypergammaglobulinemia. This generates a large panel of highly diverse Abs that do not only recognize parasite antigens, but also unrelated antigens, including self-antigens (reviewed in [191]). IgGs against brain antigens, including non-erythroid alpha spectrin, have been described in Gabonese children with cerebral malaria [559]. Some of these Abs may cross-react with parasitic antigens or neo-antigens on the iRBC surface and, in this way, contribute to the protective antiparasite response. The cysteine-rich interdomain region 1α (CIDR1α) of *Pf*EMP-1 has been identified as a mediator of T cell-independent polyclonal activation of B cells and in particular MBCs *in vitro* [710]. Direct interaction between CIDR1α and MBCs was shown to increase MBC survival and to induce MBC activation and secretion of IgM, TNF and IL-6. Parasites or parasite variants that lack the *Pf*EMP-1 CIDR1α domain such as *P. vivax* or the *Pf*EMP-1 variant VAR2CSA do not induce polyclonal B cell activation [191].

As a conclusion to section 5, attenuation of proinflammatory responses early during infection is beneficial for the parasite by promoting parasite growth and transmission, whereas later during infection it protects the host from inflammation-associated pathology. However, excessive attenuation may also be devastating for both host and parasite by causing excessive parasite growth.

6. Classification of malaria complications according to the immunological balance

Throughout this review we have outlined different mechanisms that influence the interplay between host and parasite responses. Although various balances are involved in the pathogenesis of malaria complications (Figure 1), it is the balance between the immune response of the host and the virulence of the parasite which is the main determinant of the outcome of infection (Figure 2A). Based on the current knowledge described in the previous paragraphs, we attempt to classify the different outcomes of both human and rodent malaria according to the immunological balance, as is shown in (Figure 2B and 2C).

When a sufficient antimalarial immune response is mounted early during infection and is timely downregulated to prevent excessive inflammation, disease is mild or absent. This is the case in patients with an asymptomatic or uncomplicated infection, in whom parasite replication enables parasite transmission, but without (severely) harming the host. It mostly occurs in semi-immune people in endemic regions. Such balance is also obtained in particular mouse-parasite combinations such as *P. chabaudi* AS infections in C57BL/6 mice, infections with the nonlethal strain of *P. yoelii* 17XNL or with the attenuated parasite *P. berghei* XAT.

An inefficient antiparasitic response due to insufficient immune activation and/or excessive downregulation favors parasite replication and results in hyperparasitemia. Peripheral parasitema levels can be extremely high and are accompanied by anemia due to hemolysis of iRBCs. When reticulocytosis is insufficient to alleviate anemia, suboptimal oxygen delivery may result in systemic hypoxia. Furthermore, some inflammation is induced due to the massive release of parasite and red cell components during schizont rupture. This pathology is also observed in certain mouse models of malaria, i.e. in *P. berghei* infections of mice which are Th2 prone (e.g. BALB/c), or in lethal *P. yoelii* 17XL or *P. vinckei* infections. Inefficient antiparasitic immunity in the host in combination with substantial immune evasion by the parasite results in high organ-specific parasite loads as is observed in *P. falciparum*-patients with CM or with acidosis. This may severely impair blood flow and may even cut off the blood supply to certain regions in the organ resulting in localized hypoxia and hyperlactatemia. In the brain, compensatory vasodilation may increase intracranial pressure and consequently aggravate hypoxia.

Conversely, when an exaggerated immune response is induced, inflammation causes severe pathology even if parasitemia is not maximal. This is observed in SMA with *P. falciparum* or *P. vivax* in children or mice, in whom low peripheral parasitemia levels are accompanied by increased destruction of uninfected RBCs and compromised erythropoiesis. In lungs of both patients and mice with MA-ALI or MA-ARDS, excessive pulmonary inflammation may damage the alveolar-capillary membrane, which reduces gas exchange, and in severe cases alveolar flooding may occur with subsequent suffocation. Also in other severe *P. vivax* complications, e.g. in PAM and CM, an exaggerated inflammatory response might prevail. Relatively high parasite loads may be observed in immunologically important organs such as the spleen and bone marrow, which may modulate immune reactions.

In the most severe conditions, both excessive parasite burden and excessive inflammation occur. Most typically, in murine CM, excessive immunopathological reactions involving $CD8^+$ T cells are damaging the brain and are also required for the generation of an excessive parasite burden, as depletion of pathogenic T cells results in lower parasite burden [487, 571]. Such pathogenic $CD8^+$ T cells have not been detected in patients with CM caused by *P. falciparum*. Still, immune cells and platelets sequester in the brain microvasculature and a widespread endothelial activation is evident in these patients. *P. falciparum* may use activation-induced receptors on ECs, e.g. ICAM-1, for cytoadherence and sequestration, which suggests that the parasite (mis)uses endothelial activation for evasion and proliferation [443]. It remains therefore a matter of debate whether inflammation or endothelial activation effectively plays a crucial role in the pathogenesis of human CM, and clarifying whether the observed brain swelling is due to vasogenic edema and inflammation is therefore of uttermost importance. Also PAM induced by *P. falciparum* is to be classified in the category of excessive parasite burden and excessive inflammation. Sequestration onto CSA results in high parasite loads in the placenta. As a consequence, high numbers of inflammatory cells are recruited to the placenta, which produce large amounts of inflammatory mediators resulting in tissue damage, without obtaining parasite clearance.

CONCLUDING REMARKS

A clear understanding of the balance between the immune system of the host and the virulence mechanisms of the parasite in the different malaria complications is of high relevance for the development of effective immunological interventions. A better understanding of the immunomodulatory mechanisms inhibiting long-lasting antiparasite immunity may be of critical importance for the development of more efficient vaccines or other interventions enhancing antimalarial immunity. In conditions of insufficient immunity, antiparasitic drugs are clearly the most efficient therapeutic option,

and anti-inflammatory therapy has little effect. In contrast, when exaggerated inflammation is at the basis of a severe complication of malaria, antimalarial drugs remain important to remove the etiological agent, but may be insufficient to reverse pathology. Anti-inflammatory therapy may then have beneficial effects. The definition of the type of immunological imbalance in patients with malaria complications and the development and testing of such an anti-inflammatory therapy which could be effective in these patients, however, still remain difficult challenges.

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Figure legends

Figure 1. Multiple balances determine the outcome of malaria infections.

An imbalance between host immunity and parasite evasion mechanisms during the erythrocytic phase of malaria results in pathology and may rapidly evolve into life-threatening complications. Multiple immunological, parasitological and physiological balances determine the outcome of an infection. The parasite synthesizes several immunomodulatory molecules, including glycosylphosphatidylinositols (GPIs), hemozoin (Hz) and immunostimulatory DNA. As a consequence, the host develops a prominent immune response which limits parasite proliferation. This response is based on innate immune mechanisms, e.g. phagocytic cells and natural killer (NK) cells, but in particular on antibody (Ab)-dependent phagocytosis and invasion-inhibiting Abs. These processes are orchestrated by a vast array of inflammatory mediators and leukocytes and counterbalanced by anti-inflammatory cytokines and regulatory T cells (Tregs). The counterbalance is crucial to avoid inflammation and immunopathology. Furthermore, the parasite has evolved multiple mechanisms to evade immune clearance, including sequestration, antigen variation and diversity, and may hijack the counterbalancing mechanisms of the host to downregulate immunity. Hz may also inhibit dendritic cell (DC) function resulting in impaired T and B cell responses. The erythrocyte is subject to a homeostatic balance between erythropoiesis and red cell clearance. Malaria infections greatly enhance red blood cell (RBC) clearance, not only through destruction and clearance of infected cells, but also through enhanced clearance of uninfected cells, presumably by macrophages. When this is not sufficiently compensated by erythropoiesis, severe malarial anemia may develop. Parasite sequestration may compromise the blood supply, induce hypoxia and lactic acidosis and cause metabolic disturbances. These conditions may occur in different organs, including the brain, the lungs and the placenta, and can be further aggravated by coagulation changes, e.g. as observed in disseminated intravascular coagulation (DIC), local inflammation, e.g. by releasing reactive oxygen species (ROS) and nitric oxide (NO), and immunopathological reactions that damage vascular integrity. To optimize the oxygen supply, vasodilation is triggered to increase the blood flow through the organs. These imbalances are central in a variety of complications, including cerebral malaria (CM), placenta-associated malaria (PAM), malaria-associated acute lung injury (MA-ALI)/acute respiratory distress syndrome (ARDS) and acidosis. Although these different balances play crucial roles in the pathogenesis of malaria complications, it is the balance between the immune response of the host and the virulence mechanisms of the parasite that determines whether malaria evolves to severe pathology.

Figure 2. The immunological balance.

Panel A. When a parasite is not perfectly adapted to its host, different outcomes are possible and these depend on the balance between parasite growth and immune control mechanisms. An insufficient immune response in combination with immune evasion mechanisms may lead to excessive parasite growth and a high parasite burden. This causes metabolic disturbances, severe pathology and even death. In contrast, when an exaggerated immune response is induced, the balance is tilted resulting in immunopathology and inflammation. This may also progress to severe pathology and death, albeit at a lower parasite burden. In the most severe conditions, excessive immune evasion combines with an exaggerated inflammation, resulting in both immunopathology and high parasite burden. Both inflammation and metabolic disturbances may contribute to the final pathological symptoms. In panels B and C we propose a classification of the malaria outcomes, in particular the complications, according to the immunological balance in both patients (B) and mouse models (C). The details of the balance between the parasite and the immune system is thoroughly discussed throughout the text and the classification is summarized in section 6. Question marks symbolize some important uncertainties regarding the pathogenesis of specific complications in patients.

Figure 3. Antiparasite immunity.

Phagocytes like monocytes/macrophages and dendritic cells (DCs) carry specialized receptors, e.g. CD36, which detect parasite proteins on the surface of merozoites and iRBCs. Upon phagocytosis, transcriptional activation of inflammation-associated cytokines occurs together with the generation of an oxidative burst to kill the parasites. Due to the secretion of interleukin-12 (IL-12), IL-15 and IL-18, also other cells of the innate immune system e.g. natural killer (NK) cells, γδ T cells and NKT cells become activated and start to secrete high amounts of cytokines including interferon-γ (IFN-γ) necessary to activate and enhance phagocytic processes. Furthermore, specialized antigen-presenting cells (APCs) digest and present parasite antigens in the context of major histocompatibility complex (MHC) class II (or in the context of CD1d by NKT cells for non-protein antigens), which may activate adaptive T and B cell responses. IFN-γ is produced by NK cells, γδ and αβ T cells and NKT cells. It further stimulates Th1 differentiation and the subsequent production of specific antibodies (Abs) by B cells to generate a strong antiparasite response. Opsonizing Abs together with complement deposition accelerate parasite recognition by phagocytes, whereas other Abs interfere with erythrocyte invasion and sequestration or induce intraerythrocytic parasite degeneration. Ultimately, parasites are cleared from the blood unless evasion mechanisms by the parasite, i.e. antigenic variation, alters the composition of the parasite surface

proteins. Also IL-1β, tumor necrosis factor (TNF) and IL-6 are produced mainly by phagocytic cells, which cause fever in the human host and which, when not sufficiently balanced by anti-inflammatory mechanisms, may cause collateral tissue damage. Parasite-specific $CD8^+$ T cells are also activated during infection by cross-presentation of parasitic antigens in the context of MHC class I and may cause immunopathology. ADCI, Ab-dependent cellular inhibition; ADCC, Ab-dependent cellular cytotoxicity.

Figure 4. Overview of virulence factors of a *P. falciparum*-infected RBC.

Besides intrinsic parasite growth rate and iRBC deformability, virulence is determined by a variety of factors. RBC subset tropism is in part determined by molecules of the apical complex and codetermines the parasite burden. Immunomodulating molecules released upon schizont rupture, such as heme, residual bodies containing hemozoin (Hz), uric acid precipitates, glycosylphosphatidylinositol (GPI) molecules and possibly also DNA and/or nucleosomes, are crucial factors in malaria-associated inflammation and/or immunosuppression. Variant antigen superfamilies encode parasite adhesins such as *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) and repetitive interspersed families of polypeptides (RIFINs), which mediate cytoadhesion onto endothelial cells or other RBCs, resulting in immune evasion and blood vessel obstruction. Microparticles and exosomes contribute to inflammation and intercellular communication. These virulence factors are associated with the pathophysiology of malaria. Parasite molecules, which interact directly with the host are indicated in red.

Figure 5. Imbalances result in severe pathology.

Severe complications may arise when the immune response of the host and evasion mechanisms by the parasite are not perfectly balanced. This figure illustrates two types of imbalances. (A) Excessive parasite replication may result from immune evasion and inefficient antiparasite responses. One of the major immune evasion mechanisms is cytoadherence or sequestration. Adhesion of the iRBCs to endothelial cells is mediated by binding of parasite adhesins, e.g. *Pf*EMP-1 to specific endothelial receptors, e.g. endothelial protein C receptor (EPCR), CD36, intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Rosetting through the binding of non-infected RBCs to infected RBCs is also mediated by parasite adhesins (e.g. RIFINs) and contributes to vessel obstruction. This leads to high organ-specific parasite loads and may severely impair the blood flow or may even cut off the blood supply to specific areas in the organ resulting in localized hypoxia and hyperlactatemia. Furthermore, inflammation is induced due to the massive release of parasite and red cell components during schizont rupture and by activated platelets. Together with the production of hypoxia-inducible proteins, e.g. vascular endothelial growth factor (VEGF), this may alter vascular permeability. (B) An

exaggerated inflammatory response, e.g. due to insufficient anti-inflammatory regulation, also causes also severe pathology. Chemokines, including monocyte chemotactic protein-1 (MCP-1)/CCL2 and interferon gamma inducible protein-10 (IP-10)/CXCL10 attract monocyte/macrophages, NK cells, $CD4^+$ and $CD8^+$ T cells which adhere to the microvascular lining by interaction with specific adhesion molecules, e.g. P-selectin, ICAM-1 and VCAM-1. Leukocyte sequestration and excessive release of proinflammatory or cytotoxic mediators such as tumor necrosis factor (TNF), interferon-γ (IFN-γ) and perforin/granzyme, result in blood flow perturbations and endothelial damage. Sequestration of iRBCs may further aggravate this process, and accumulation of iRBCs may be enhanced by leukocyte-induced vascular obstruction and endothelial expression of adhesion molecules. Adhering platelets provide additional receptors for sequestration and secrete chemokines, e.g. platelet factor 4 (PF4/CXCL4), cytokines, e.g. transforming growth factor-β (TGF-β) and other factors, e.g. VEGF, and fuel both vascular obstruction by parasite sequestration (panel A) and inflammation (panel B). Furthermore, high amounts of microparticles (MPs) of different origins, e.g. from iRBCs and platelets, are produced and may transfer platelet and parasite proteins to the endothelial cell surface, promoting the inflammatory and procoagulant state of the endothelium. Excessive parasite replication (panel A) and exaggerated inflammation (panel B) may occur separately or simultaneously and cause life-threatening complications (see also Figure 2).

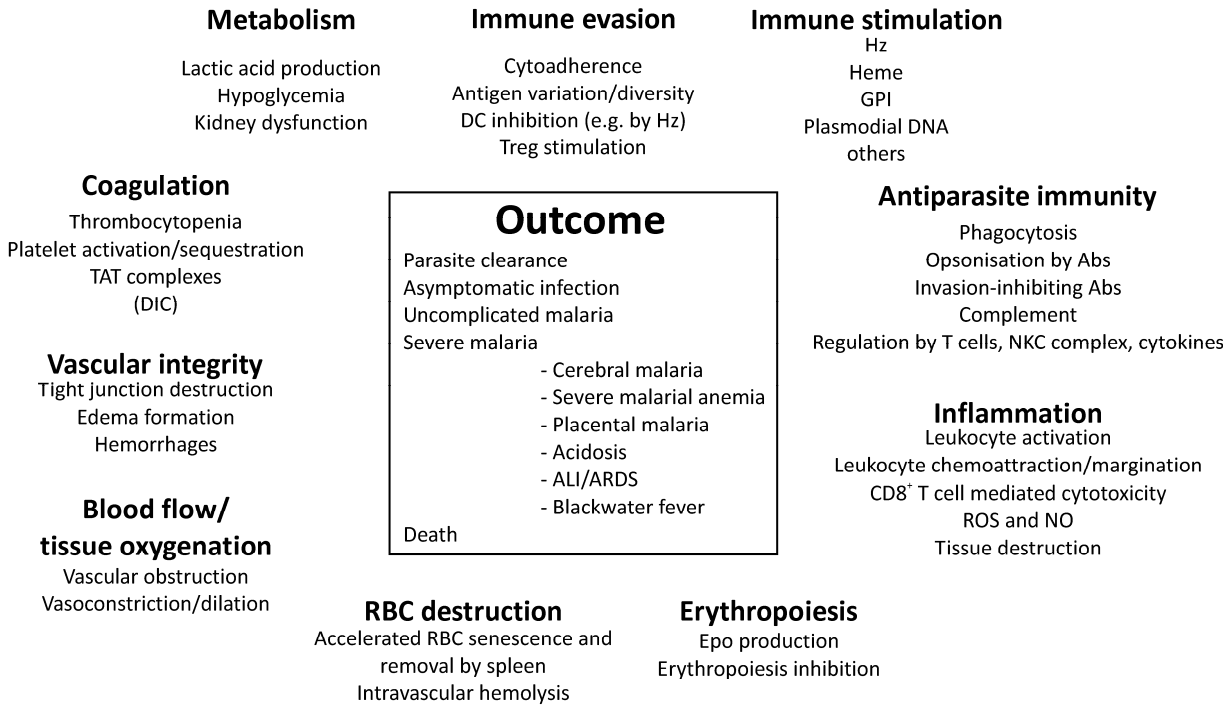

Metabolism
Lactic acid production
Hypoglycemia
Kidney dysfunction
Immune evasion
Cytoadherence
Antigen variation/diversity
DC inhibition (e.g. by Hz)
Treg stimulation
Immune stimulation
Hz
Heme
GPI
Plasmodial DNA
others
Coagulation
Thrombocytopenia
Platelet activation/sequestration
TAT complexes
(DIC)
Outcome
Parasite clearance
Asymptomatic infection
Uncomplicated malaria
Severe malaria
- Cerebral malaria
- Severe malarial anemia
- Placental malaria
- Acidosis
- ALI/ARDS
- Blackwater fever
Death
Antiparasite immunity
Phagocytosis
Opsonisation by Abs
Invasion-inhibiting Abs
Complement
Regulation by T cells, NKC complex, cytokines
Vascular integrity
Tight junction destruction
Edema formation
Hemorrhages
Inflammation
Leukocyte activation
Leukocyte chemoattraction/margination
CD8+ T cell mediated cytotoxicity
ROS and NO
Tissue destruction
Blood flow/
tissue oxygenation
Vascular obstruction
Vasoconstriction/dilation
RBC destruction
Accelerated RBC senescence and
removal by spleen
Intravascular hemolysis
Erythropoiesis
Epo production
Erythropoiesis inhibition

Figure 1

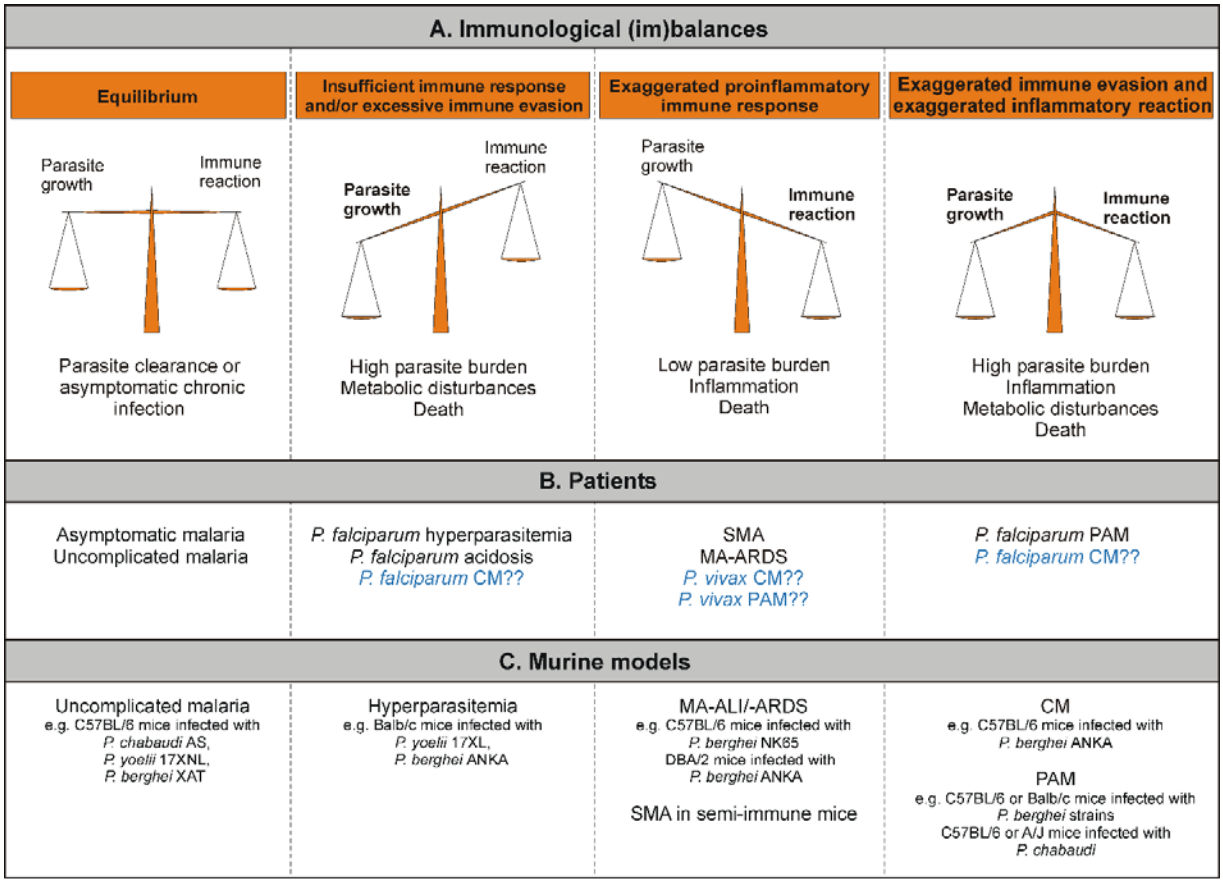

A. Immunological (im)balances
Equilibrium
Insufficient immune response and/or excessive immune evasion
Exaggerated proinflammatory immune response
Exaggerated immune evasion and exaggerated inflammatory reaction
Parasite growth
Immune reaction
Parasite clearance or asymptomatic chronic infection
High parasite burden
Metabolic disturbances
Death
Low parasite burden
Inflammation
Death
High parasite burden
Inflammation
Metabolic disturbances
Death
B. Patients
Asymptomatic malaria
Uncomplicated malaria
P. falciparum hyperparasitemia
P. falciparum acidosis
P. falciparum CM??
SMA
MA-ARDS
P. vivax CM??
P. vivax PAM??
P. falciparum PAM
P. falciparum CM??
C. Murine models
Uncomplicated malaria
e.g. C57BL/6 mice infected with
P. chabaudi AS,
P. yoelii 17XNL,
P. berghei XAT
Hyperparasitemia
e.g. Balb/c mice infected with
P. yoelii 17XL,
P. berghei ANKA
MA-ALI/-ARDS
e.g. C57BL/6 mice infected with
P. berghei NK65
DBA/2 mice infected with
P. berghei ANKA
SMA in semi-immune mice
CM
e.g. C57BL/6 mice infected with
P. berghei ANKA
PAM
e.g. C57BL/6 or Balb/c mice infected with
P. berghei strains
C57BL/6 or A/J mice infected with
P. chabaudi

Figure 2

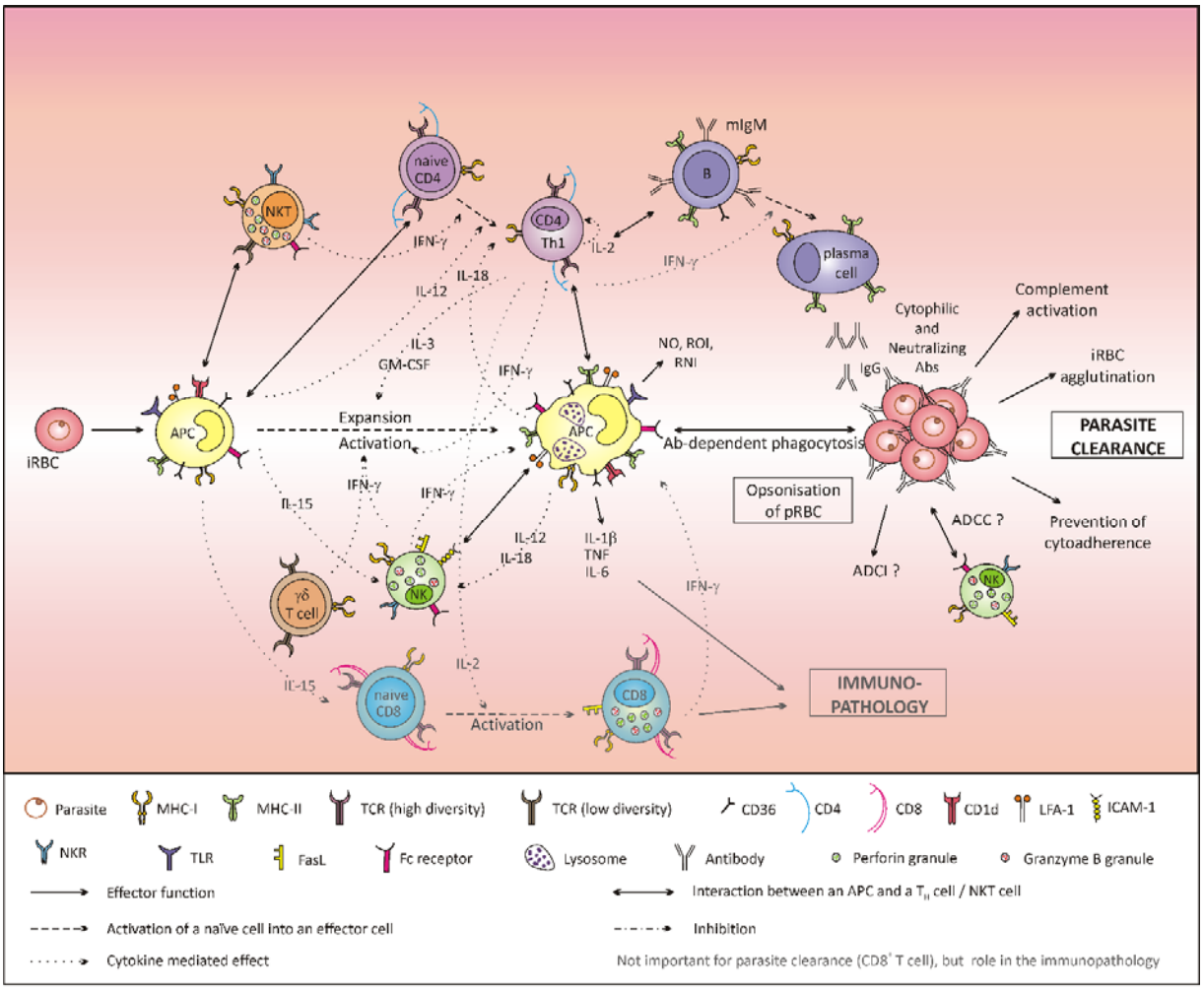

naive CD4
NKT
CD4 Th1
B
mIgM
plasma cell
IFN-γ
IL-2
IL-12
IL-18
IL-3
GM-CSF
NO, ROI, RNI
Cytophilic and Neutralizing Abs
IgG
Complement activation
iRBC agglutination
PARASITE CLEARANCE
iRBC
APC
Expansion
Activation
Ab-dependent phagocytosis
Opsonisation of pRBC
ADCC ?
ADCI ?
Prevention of cytoadherence
IL-15
IL-1β
TNF
IL-6
γδ T cell
NK
naive CD8
CD8
Activation
IMMUNO-PATHOLOGY
Parasite
MHC-I
MHC-II
TCR (high diversity)
TCR (low diversity)
CD36
CD4
CD8
CD1d
LFA-1
ICAM-1
NKR
TLR
FasL
Fc receptor
Lysosome
Antibody
Perforin granule
Granzyme B granule
Effector function
Interaction between an APC and a T_H cell / NKT cell
Activation of a naïve cell into an effector cell
Inhibition
Cytokine mediated effect
Not important for parasite clearance (CD8+ T cell), but role in the immunopathology

Figure 3

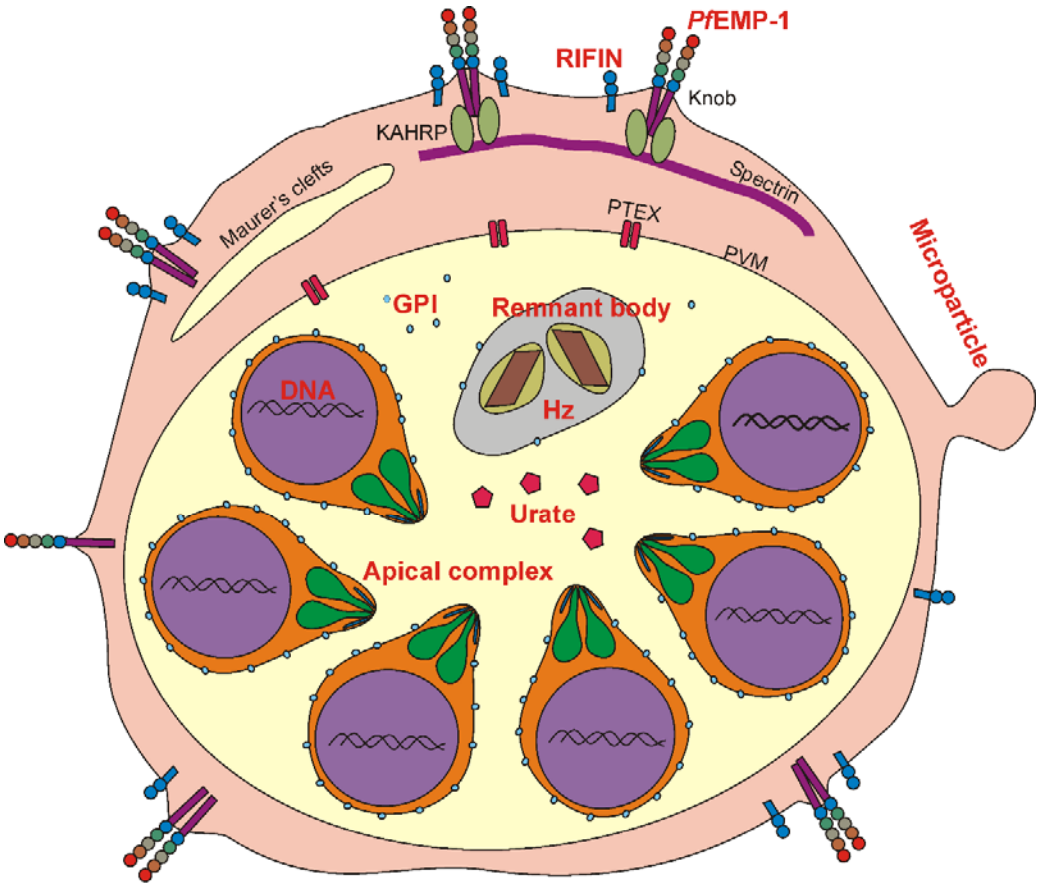

PfEMP-1
RIFIN
Knob
KAHRP
Spectrin
Maurer's clefts
PTEX
PVM
Microparticle
GPI
Remnant body
DNA
Hz
Urate
Apical complex

Figure 4

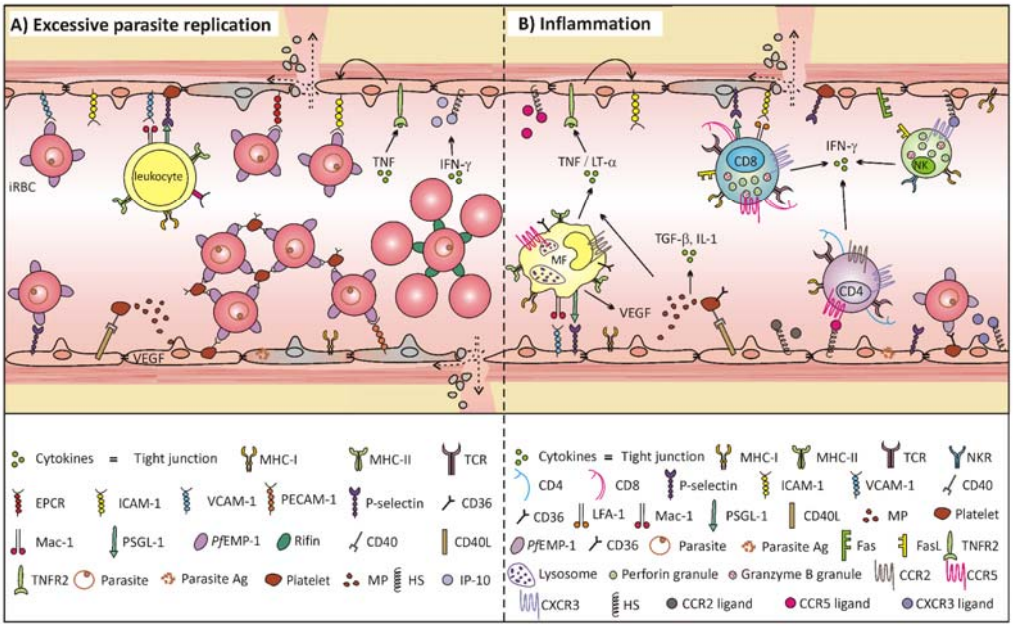
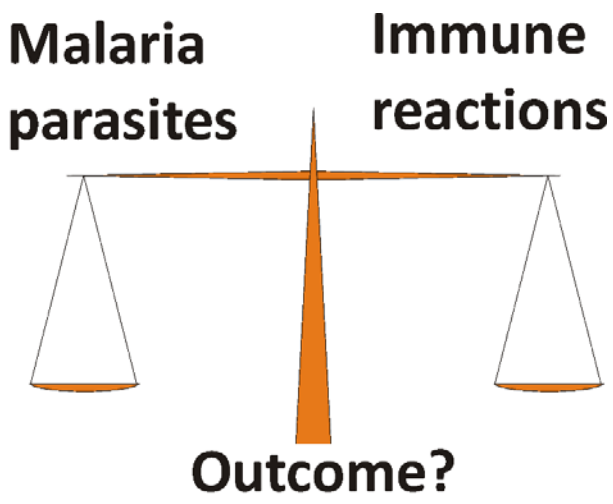

A) Excessive parasite replication
B) Inflammation
iRBC
leukocyte
TNF
IFN-γ
VEGF
TNF / LT-α
CD8
IFN-γ
NK
MF
TGF-β, IL-1
VEGF
CD4
Cytokines
Tight junction
MHC-I
MHC-II
TCR
NKR
CD4
CD8
P-selectin
ICAM-1
VCAM-1
CD40
EPCR
PECAM-1
CD36
LFA-1
Mac-1
PSGL-1
CD40L
MP
Platelet
PfEMP-1
Rifin
Parasite
Parasite Ag
Fas
FasL
TNFR2
Lysosome
Perforin granule
Granzyme B granule
CCR2
CCR5
CXCR3
HS
IP-10
CCR2 ligand
CCR5 ligand
CXCR3 ligand

Figure 5



Graphical Abstract